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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Report on a Collaborative Study of the Assay of Ergot*

By RALPH G. SMITH†

This report presents a record of the results of a collaborative study of the assay of ergot extending over a period of several years. A colorimetric method is recommended by which ergot and ergot preparations are assayed for total alkaloids and for ergonovine.

FOLLOWING the discovery of ergonovine and the demonstration of its importance as an oxytocic agent it soon became apparent that the available methods for the assay of galenical preparations of ergot were inadequate. These methods either did not measure the ergonovine content of ergot or did not distinguish it from other alkaloidal constituents which were less active on the uterus and which were present in larger amounts (1). Consequently, in the fall of 1940 a Subgroup of the Subcommittee on Biological Assays of the United States Pharmacopœia was appointed to undertake a study of the assay of ergot and, if possible, to make a recommendation of a method suitable for adoption by the United States Pharmacopœia. The membership of the original Subgroup was as follows:

Walter L. Mendenhall, Boston University Medical School
J. Allen Reese, University of Kansas
E. E. Swanson, Lilly Research Laboratories
Marvin R. Thompson, Warner Institute for Therapeutic Research
Ralph G. Smith (Chairman), University of Michigan.

Additional collaborators (cited below) with experience in this field of investigation gave valuable assistance in the studies which followed. After it was decided in 1945 that ergot and fluidextract of ergot be not admitted to U. S. P. XIII, these preparations were included in the Eighth Edition of the National Formulary. Consequently, it was agreed by all parties concerned that the results of the collaborative study should be made available to the Committee on the National Formulary. The following is a report on the collaborative study of the assay of ergot conducted by this Subgroup.

An informal conference of those interested in the assay of ergot was held in Chicago, April 18, 1941, for the purpose of outlining the problem and making general plans for

* Received July 7, 1947.
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the project. It was agreed that ergot should be assayed for both its ergonovine and total alkaloidal content which would involve the extraction and separation of ergonovine from the other alkaloids. Published methods involving such a procedure were considered unsatisfactory and extensive collaborative assays were considered inadvisable until a reasonably satisfactory method was available. Three of those present, Donald C. Grove, Federal Food and Drug Administration, C. A. Morrell, Food and Drug Laboratory of the Department of National Health and Welfare, Ottawa, Canada, and E. E. Swanson, Lilly Research Laboratories, each outlined a tentative method for the extraction and separation of alkaloidal fractions. It was agreed that the three methods involving both colorimetric and biological assays should be tested and compared on a common sample of powdered ergot by the above three men and the chairman. The assays were conducted accordingly and served to clarify certain technical difficulties in the procedures. The results of these preliminary trials are not reported since the details of technique employed by the collaborators were not sufficiently uniform to make them comparable.

A second meeting of the Subgroup was held in Detroit, on August 21, 1941. It was the consensus of those present that an adequate assay of ergot and the fluidextract of ergot would be constituted by a colorimetric assay for total alkaloids and after separation of the water-soluble fraction a bioassay or possibly a colorimetric assay for ergonovine. Since the water-insoluble alkaloids were considered to be of minor importance in the therapeutic use of the drug, it was believed that a colorimetric assay for total alkaloids would suffice even though it also measured the biologically inactive isomers. Furthermore a colorimetric assay would be preferable from the standpoint of simplicity of procedure and of accuracy. With respect to ergonovine a bioassay was considered necessary unless it could be shown that the extraction and separation procedures would yield a product for which the results of the bioassay and colorimetric assay were the same. It was planned to compare four procedures for the extraction and separation of the alkaloids on a common sample of ground, dried defatted ergot supplied by the Eli Lilly Company and distributed to the collaborators through Dr. E. Fullerton Cook, Chairman of the U. S. P. Committee of Revision. Such a sample would permit a comparison of the methods on a preparation, uniform with respect to grinding and defatting and more stable than a ground nondefatted ergot (2). It was decided that the bioassay of ergonovine should be conducted on the isolated rabbit uterus but the details of procedure were not specified at this time nor were the details for the colorimetric procedure. U. S. P. Ergotoxine Ethanesulfonate Reference Standard was used as a standard for the colorimetric assay for total alkaloids. Pure ergonovine maleate supplied by the Eli Lilly Company was used as a standard for the colorimetric and bioassay for ergonovine.[1]

METHODS

The four methods for the extraction and separation of the alkaloids are described below. These include the three procedures (with minor modifications) used in the previously mentioned preliminary trials and a fourth method submitted by the chairman. Some of these have been published as indicated.

Method I, *submitted by Donald C. Grove* (3)

Accurately weigh 10 Gm. of the defatted ergot in a 100-cc. beaker. Add 8 cc. of lead subacetate solution (N. F. V strength approx. 22.5% Pb) and mix intimately with a stout glass rod until the drug is thoroughly moistened. Transfer the mixture to a paper extraction thimble, 33 × 80 mm., brushing out any of the powder remaining in the beaker with a small camel's hair brush. Place a small pledget of cotton over the drug in the thimble and extract the drug for four hours on the steam bath in an automatic extractor of the type shown in Ace Class, Inc., Catalog No. 6725, size C, using about 100 cc. of U. S. P. XI anesthetic ether. Transfer the ether extract to a 125-cc. separator, rinsing the flask several times with small portions of ether and finally

[1] At this meeting the following actions were also taken:

[a] Since the fluidextract of ergot is an unstable product and no preparation is available which will ensure that it reaches the customer in a state which meets the present assay requirements it is recommended that the part of the (U. S. P.) monograph referable to the assay of the fluidextract of ergot in the Eleventh Decennial Revision of the United States Pharmacopœia be deleted from the Twelfth Revision.

[b] In view of the above, it is also recommended that defatted ergot powder, which is a relatively stable preparation, be substituted for the Fluidextract of Ergot as a dosage form in the Twelfth Revision of the United States Pharmacopœia.

with 10 cc. of 1% tartaric acid. Shake the alkaloids out of the ether with 1% tartaric acid, using one 10-cc. portion and six successive 5-cc. portions. Combine the acid extracts in a 100-cc. beaker and remove the ether on the steam bath at a temperature of about 40°, using an air blast. Transfer the ether-free acid extract to a 50-cc. volumetric flask and make to volume with water. Remove a 10-cc. aliquot (representing 2 Gm. of drug) and dilute to a suitable volume for the colorimetric estimation of *total alkaloids*. Wash the pipette used to remove this aliquot with about 2–3 cc. water, adding the washings to the remaining 40 cc. of acid extract. Add ammonia (10%) until the mixture is just alkaline to litmus and make to volume with water. Filter and place a 25-cc. aliquot (representing 4 Gm. of drug) of the clear filtrate into a 125-cc. separator. (The following procedure, based on the differential distribution of ergonovine and ergometrinine between ether and water, is designed to separate the latter alkaloid from ergonovine. The results for the colorimetric and biological assays should thus be identical.) Add 35 cc. U. S. P. ether and shake the mixture for three minutes. It is essential that this period of shaking be used in making all of the extractions throughout this procedure. Invert the separator and cautiously open the stopcock to permit the release of excess pressure. Allow the two layers to separate sharply and draw off the water layer as completely as possible into a second separator containing 35 cc. of ether. Shake the second separator and withdraw the water layer into a third separator containing 35 cc. of ether. After shaking in the third separator, withdraw the water into a 250-cc. separator. Repeat the above described process with six successive 10-cc. portions of weak ammonia water (prepared by adding one drop of 10% NH_3 *w/v* to 200 cc. water) and combine the water extracts in the 250-cc. separator. Saturate the water extracts with NaCl (about 30 Gm.) and extract the alkaloid with six successive 10-cc. portions of $CHCl_3$, filtering each extract through a funnel with a small pledget of cotton, previously moistened with chloroform, into a 100-cc. beaker. Evaporate the combined chloroform extracts to dryness with an air blast at a temperature of about 40°. The residue is dissolved in small portions of water and made to 25 cc. or other suitable volume. Usually the residue has a somewhat greasy appearance and it is necessary to rub it well with the water, using a rubber policeman, when it gradually goes into solution. The water solution is then determined by both the colorimetric and bioassay procedures. If only the colorimetric method is used, the last step—saturating with salt and extracting with chloroform—can be eliminated.

Method II, *submitted by C. E. Powell, O. W. Reagan, Asa Stevens, and Edward E. Swanson* (4)

Place 12.5 Gm. of the ground defatted crude drug in a flask suitable for use in a mechanical shaker. Add 125 cc. of reagent acetone plus 2.5 cc. of 10% ammonia, shake for one hour, filter, take 100 cc. of the filtrate, which represents 10 Gm. of the drug, and evaporate under a current of air to approximately 25 cc. Add 10 cc. of distilled water plus enough tartaric acid to produce a *p*H of 3. Shake the acid-acetone-water solution with two 50-cc. portions of ether to remove the fats and most of the coloring matter, and evaporate off the last traces of ether by vacuum distillation. Test this solution for total alkaloids colorimetrically. Make the solution alkaline to a *p*H of 8 with sodium bicarbonate, allow to stand for ten minutes, filter, and wash the container and filter paper with two 10-cc. portions of a 1% sodium bicarbonate solution. The precipitate on the filter paper represents most of the *water-insoluble alkaloids* (*ergotoxine-like*) and the filtrate contains the *water-soluble alkaloid* (*ergonovine*). Dissolve the precipitate on the filter paper in 10 cc. of a 2% solution of tartaric acid and determine the ergotoxine-like alkaloids colorimetrically.

In a separatory funnel, shake the filtrate (ergonovine) with two 50-cc. portions of carbon tetrachloride (ten minutes for each shaking) to remove the last traces of the ergotoxine-like alkaloids, run off the carbon tetrachloride, and remove all traces of carbon tetrachloride from the ergonovine solution by vacuum distillation (a little heat may be necessary). Add sodium chloride to saturation and shake with successive portions of ether until there is no blue color (*p*-dimethylaminobenzaldehyde reagent). Evaporate off the ether by air current into 10 cc. of distilled water made slightly acid (*p*H 3 or 4) with tartaric acid and assay biologically and colorimetrically with ergonovine maleate as a standard.

Method III, *submitted by W. M. Bachinski, M. G. Allmark, and C. A. Morrell*

Eight grams of the defatted ergot powder is accurately weighed and transferred to a glass-stoppered 125-cc. Erlenmeyer flask. Ten cubic centimeters of the U. S. P. XI alcohol-HCl menstruum per gram of powder is then added, the flask stoppered tightly and placed in a mechanical shaker with a to-and-fro motion. The mixture is shaken for one hour, and then centrifuged, using large centrifuge cups. The supernatant liquid is poured into an Erlenmeyer flask and subjected to reduced pressure for about one minute to remove air bubbles. A 5-cc. portion of the macerate is placed in a Watkins extractor (type I) (see Fig. 1). One cubic centimeter of dilute NH_3 B. P. and about 0.01 Gm. powdered MgO, and 8 cc. of water are added. The magnesium oxide serves a double purpose: it helps to break up emulsions and also serves to keep the mixture alkaline. Glass wool may be placed around the inner funnel near the surface of the mixture if emulsions occur. A little powdered porcelain with 100 cc. of ether is placed in a 125-cc. Erlenmeyer flask, attached to the extractor, and the flask is placed in a constant temperature water bath kept at 50°. A reflux condenser is attached to the Watkins extractor. The extraction is carried on for

two hours. (Work done in this laboratory has shown that using the type of apparatus specified and the conditions described, the total alkaloids are removed in two hours. About 95% is removed in one hour.)

The ether extract is then extracted by shaking in a separatory funnel with six 10-cc. portions of 1% tartaric acid, and the combined tartaric acid extracts warmed to remove dissolved ether and to reduce the volume; solution is then made up to 50 cc. in a volumetric flask. The total alkaloids are determined colorimetrically, using an Evelyn photoelectric colorimeter with filter number 580. A visual colorimeter may also be used.

Fifty cubic centimeters of the macerate are placed in a large capacity Watkins extractor (type II) (see Fig. 1). Five cubic centimeters of dilute

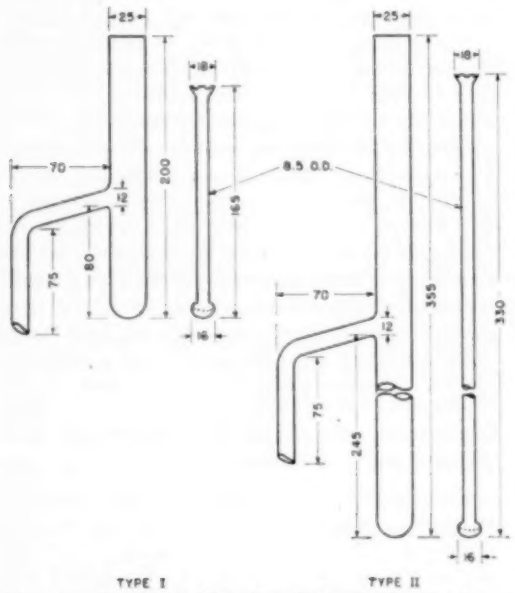

Fig. 1.—Modified Watkins Extractors. Scale: 1 inch = 132 mm.

NH_3 B. P. and about 0.20 Gm. of magnesium oxide are added, and extracted for *six hours* (it is possible that this time may be shortened to four or even two hours and the problem is being further investigated) at 50° using 200 cc. U. S. P. ether with a little powdered porcelain in a 250-cc. Erlenmeyer flask. After about four hours of extraction, 6 to 7 cc. of water is added to the brown mixture in the extractor. Glass wool may be necessary to prevent gel from coming over into the flask. The ether extract is evaporated to suitable volume, then shaken out with seven 10-cc. portions of 1% tartaric acid. The tartaric acid extract is warmed to remove the ether and to reduce the volume, finally being made up to 50 cc. The total alkaloids are determined colorimetrically after suitable dilution. The value secured should agree with that obtained at the earlier part of the procedure, otherwise extraction has been incomplete.

Ergonovine is determined using Grove's procedure [(3) and see above]. A 30-cc. aliquot of this solution of alkaloids is placed in a beaker, 2 cc. of 10% NH_3 is added, and the mixture let stand 15 minutes before filtering. Twenty-five cubic centimeters of the filtrate is used in Grove's method. Ergonovine is determined colorimetrically, and biologically by evaporating the water extract obtained to about 20 cc. and using ergonovine acid maleate as the standard.

As an alternative, ergonovine plus ergometrinine may be determined by the precipitation of the ergotoxine alkaloids by phosphotungstic and phosphomolybdic acid after the total alkaloids are in the tartaric acid solution. If anyone wishes to use this method, a copy of the manuscript by Morrell and Chapman containing the details of procedure may be obtained.

Method IV, *submitted by Ralph G. Smith*

The following procedure utilizes certain features of the method of Swanson and co-workers and of the method of Grove. Acetone is used for the initial extraction as it seems to be more efficient than ether when both are used under the same conditions, although the former solvent does remove more color. The method avoids the loss of alkaloid which occurs in the Swanson method when the acid-acetone-water solution is washed with ethyl ether to remove color. The adoption of the Grove ether-water distribution procedure should yield ergonovine alone rather than ergonovine and ergometrinine.

Place 12 Gm. of powdered defatted ergot in a 250-cc. flask. Add 118 cc. of reagent acetone and 2 cc. of a 10% solution of ammonia. Stopper the flask and shake it in a mechanical shaker for one hour. Filter and transfer 100 cc. of filtrate, which represents 10 Gm. of the defatted ergot, to an evaporating dish. Evaporate it at room temperature under an air current to approximately 20 cc. Measure the volume and transfer the solution to a 125-cc. separatory funnel. Add approximately 3 volumes of ethyl ether, using it to wash the evaporating dish and measuring cylinder. This acetone-ether mixture will form a layer over water. Make acid with 0.2 cc. of 20% tartaric acid. Extract with four 10-cc. samples of 1% tartaric acid solution, in each case drawing off the aqueous solution into a small round-bottomed flask. From the combined samples remove traces of ether and acetone by evacuation at approximately 40°. Transfer the solution to a 50-cc. volumetric flask, washing with small volumes of 1% tartaric acid to make to volume. Henceforth, the procedure is as described in the Grove method [(3) and see above], removing a 10-cc. aliquot (representing 2 Gm. of drug) for colorimetric estimation of total alkaloids to be expressed as ergotoxine or ergotoxine ethanesulfonate and continuing from that point in his description.

TABLE I.—SUMMARY OF RESULTS: FIRST ASSAY OF DEFATTED ERGOT POWDER NO. 290643

Results for total alkaloids are expressed as mg. of ergotoxineethanesulfonate per Gm. of defatted ergot powder. Results for ergonovine are expressed as mg. of ergonovine maleate per Gm. of defatted ergot powder.

Where two results were recorded in the original report, averages were used in this summary.

Method No.	Report No.	Total Alkaloids Colorimetric	Ergonovine	
			Colorimetric	Bioassay
I	1	3.27	0.23	0.22
	2	3.23	0.17	0.17
	3	3.00	0.11	0.12
	4	3.42	0.21	0.17
	5	2.94	0.15	...
	6	3.29	0.28	...
	Av.	3.19	0.19	0.17
		S. D. ±0.18	S.D. ±0.06	S. D. ±0.04
II	1	2.71	0.26	0.14
	2	2.73	0.14	0.12
	3	2.24	0.12	0.12
	4	3.28	0.18	0.14
	5	2.93	0.24	...
	6	2.54	0.05	...
	Av.	2.74	0.165	0.13
		S. D. ±0.35	S. D. ±0.08	S. D. ±0.01
III	1	3.06	0.30	0.18
	2	3.24	0.20	0.21
	3	2.46	0.09	0.09
	4	3.17	...	...
	5	...	...	...
	6	2.88	0.16	...
	Av.	2.96	0.19	0.16
		S. D. ±0.31	S. D. ±0.09	S. D. ±0.06
IV	1	3.24	0.22	0.16
	2	3.10	0.14	0.15
	3	2.62	0.11	0.12
	4	3.51	0.23	0.18
	5	2.82	0.20	...
	6	2.64	0.19	...
	Av.	2.99	0.18	0.15
		S. D. ±0.36	S. D. ±0.05	S. D. ±0.025

(Chloroform has not yet been used for the extraction of ergonovine from the saturated aqueous solution of sodium chloride. Instead, four 100-cc. samples of anesthetic ether have been used for this purpose as described in the Swanson method. In a personal communication, Dr. Grove has pointed out that chloroform extracts ergonovine from an aqueous solution more readily than does ether as shown by Hampshire and Page (5) and also by Schou and Bennekou (6). It also is more convenient in that it forms the bottom layer in the separatory funnel.)

For various reasons the project was delayed until the summer of 1943 when the descriptions of the methods, standards, and sample of ergot were supplied to those who wished to collaborate. Six reports were submitted by the following:

Donald C. Grove and Bert J. Vos, Federal Food and Drug Administration Laboratories

W. M. Bachinski, M. G. Allmark, and C. A. Morrell, Food and Drug Laboratory, Department of National Health and Welfare, Ottawa, Canada

C. E. Powell, O. W. Reagan, and Edward E. Swanson, Lilly Research Laboratories

J. Allen Reese, School of Pharmacy, University of Kansas

L. W. Rowe, Research and Biological Laboratories, Parke, Davis and Company

Ralph G. Smith, Department of Pharmacology, University of Michigan and Tulane University

The results are recorded in Table I.[2]

Comments on the methods, submitted by the collaborators, are summarized as follows:

"From the standpoint of the amount of time consumed in these assay methods, Method II is the quickest; however, this method in our hands does not show very good agreement between the colori-

[2] Dr. Reese reported the following results obtained colorimetrically by the method of Allport and Jones (7). It may be noted that the values for total alkaloids are in the range of those obtained by the above four methods but the values for ergonovine are definitely higher.

	By Acetone Extraction	By Ether Extraction
	Expressed as mg. ergotoxine ethanesulfonate per Gm. of ergot	
Total alkaloids	2.94	2.86
Ergotoxine	2.18	2.13
Ergonovine	0.48	0.47
	Expressed as mg. ergonovine acid maleate per Gm. of ergot	
Ergonovine	0.29	0.28

metric and bioassay for ergonovine, and the value for total alkaloids is lower.

"Method IV is next with regard to speed of operation and, while the colorimetric values for ergonovine agree fairly well with those obtained by Method I, the bioassay results are lower although not as low as those obtained by Method II.

"Method III is just about as long as Method I, and it gave a rather high colorimetric value for ergonovine and a low bioassay; however, the number of milligrams of ergonovine found by bioassay was higher than that obtained by either Method II or IV.

"The agreement by Method I between the colorimetric and bioassay was extremely close."

"In Method II the final 50-cc. tartaric acid solution containing the total alkaloids was yellowish brown in color, although when diluted for colorimetric assay it was water-clear. The loss in total alkaloids probably occurred in the stage when the acid-acetone-water mixture was washed twice with 50-cc. portions of ether. The sludge contained in this ether extract might have adsorbed material difficult to re-extract for chemical examination. This seems to have been overcome in Method IV. In Method I the final 50-cc. tartaric acid solution of total alkaloids was oily in appearance, yellow, and slightly opaque. However, when diluted for colorimetric test, the solution was much clearer.

"The Grove procedure for ergonovine was found to be satisfactory, giving almost water-clear solutions of ergonovine for bioassay."

"According to my results, total alkaloids may be determined satisfactorily by any of the three methods. However, there is quite a discrepancy in the ergonovine fraction."

"By the Methods I and III, in making the final aqueous solutions of ergonovine there is more fatty material in the residue after the chloroform extraction than in Methods II and IV where ether is used. Consequently more time is necessary in making the aqueous solution to insure complete solution of the ergonovine. It is questionable whether the theoretical advantage of chloroform over ether justifies its use. Some delay was also caused by emulsion formation in this final chloroform extraction.

"Method II takes less time than the other methods although the results are somewhat lower."

"In our hands, the above results indicate that each of the four methods is equally proficient in estimating the ergonovine activity of the crude drug.

"There is, however, a slight difference in the total alkaloidal content which we are unable to explain. We believe, therefore, that the results obtained by the four methods agree reasonably well. From the standpoint of simplicity and time saving, Method II can be carried out in duplicate and completed in one day, whereas the other three methods require more apparatus and two days for completion.

"It has been shown (4) that CCl_4 removes considerable colorimetrically active material that has only a weak physiological action. Since the final results of all methods agree so closely in the ergonovine estimation of the crude drug, it is our opinion that two CCl_4 shakings accomplish the same results as the 21 shakings required by the Grove procedure (3) to eliminate the ergometrinine and other inactive alkaloids."

In September, 1944, a third meeting was held in Cleveland to discuss the results of the first collaborative assay and to plan for further studies if such were considered advisable. At this meeting it was the consensus that any one of the four extraction methods was reasonably satisfactory. Since the therapeutic potency of the ergonovine fraction of ergot could be most reliably measured by a bioassay and since a constant relation between the results of the colorimetric and biological assays was not evident (Table I) the adoption of a colorimetric rather than a bioassay was considered to be still unjustified.

In order to secure further data it was decided to carry out the following program:

(*a*) A second collaborative assay would be made on three samples of ergot and on three fluidextracts of ergot prepared from these samples. Defatted ergot powder No. 290643 used in the previous study would serve as one sample. The two other samples would be supplied by the U. S. P. office, one of these to be preferably an ergot of low potency.

(*b*) Assays would be made for total alkaloids colorimetrically and for ergonovine colorimetrically and biologically.

(*c*) In order to limit the labor involved two extraction methods for ergot and for the fluidextract would be used, one to be described by Dr. Grove and one by Dr. Smith. The choice of the two methods was made to some extent arbitrarily. However, it was believed that Method I should be used again since the results (Table I) obtained by this method showed close agreement between the colorimetric and bioassay results for ergonovine. It included the Grove procedure (3) which should theoretically give such results. Also the values obtained for total alkaloids indicated a more complete extraction of ergot than by the other methods. Method I, with modifications suggested by experience with it, is designated here as Method V. The other method described by Smith was modified from Method IV by the elimination of the Grove procedure for the separation of ergometrinine from ergonovine and followed more closely Method II in that it included the washing of the water-soluble fraction with carbon tetrachloride. The object was to provide a procedure which might prove to be satisfactory and would certainly be less laborious than Method I, III, or IV. Furthermore, the manner in which the procedure differed from Method II resulted in a higher yield of total alkaloids (Table I), comparable with those obtained by the more time-consuming methods. The procedure is designated here as Method VI. The descriptions of the methods for this second collaborative assay also included procedures for the fluidextract of ergot. Results obtained by the official method of the new British Pharmacopœia would also be considered if collaborators wished to employ it. It was doubted, however, that the extract obtained by this method could be used for the bioassay of ergonovine.

(*d*) Dr. Grove agreed to outline details for the colorimetric procedure in order that all collaborators would use the same technique.

(*e*) It was decided to use the method of Vos (8) for the bioassay of ergonovine.

Method V, ***submitted by Donald C. Grove***

Ergot or Defatted Ergot.—Ten grams of pow-

dered ergot is weighed into a 100-cc. beaker, 4 cc. of lead subacetate solution (approximately 22.5% Pb) is added, the mixture thoroughly mixed with a glass rod and transferred to a 33 × 80-mm. paper extraction thimble, using a camel's hair brush to remove small amounts of powder remaining in the beaker. A small pledget of cotton is placed over the drug in the thimble and the thimble placed in an extraction flask (see Catalog, Ace Glass, Inc., No. 6725, size C). (It has been found advantageous to have a small side arm blown into the flask. This side arm enables one to transfer the ether extract into the separator more easily.) One hundred cubic centimeters of ether is poured through the thimble, the flask connected to the condenser, and the drug extracted for four hours on a steam bath.

The ether extract is transferred to a 125-cc. separator, the flask rinsed several times with small portions of ether, and then with two 5-cc. portions of 1% tartaric acid solution. This gives 10 cc. of 1% tartaric acid which is used for the first shake-out of the ether and is followed by six successive 5-cc. portions of 1% tartaric acid. Generally after about the third shake-out some emulsification occurs. This can be eliminated by adding 1 cc. of alcohol after the third shake-out and 1 cc. more of alcohol after the fifth shake-out. The tartaric acid extracts are combined in a long-neck, 200-cc. round-bottomed flask which is connected to a vacuum line or to a water pump and the ether removed at room temperature. The solution is then transferred to a 50-cc. volumetric flask with the aid of small portions of distilled water and made up to 50 cc. At this point the solution may be kept overnight in a refrigerator and the assay completed the next day.

A 5-cc. aliquot is pipetted out of the 50 cc. of tartaric acid solution of the alkaloids and made up to 50 cc. with distilled water. This solution is then reserved for the colorimetric determination of total alkaloids and represents the alkaloids from 1 Gm. of drug in 50 cc. The 5-cc. pipette is rinsed with about 2 cc. of distilled water and the rinsing returned to the 45 cc. of tartaric acid solution. Ammonia water (10%) is then added to this solution until the water-insoluble alkaloids have precipitated and the solution is alkaline to litmus, the mixture then being diluted to 50 cc. with distilled water.

The mixture is filtered and a 45-cc. aliquot of the clear filtrate is pipetted into a 125-cc. separator. One-half gram of powdered tartaric acid, 10 Gm. of NaCl, and 50 cc. of washed ether (400 cc. ether washed three times with 100-cc. portions of distilled water to remove alcohol and filtered through purified cotton) are added and the mixture thoroughly shaken. (This step removes a slight amount of coloring material.) The aqueous layer is withdrawn into another 125-cc. separator and the ether washed with two 5-cc. portions of saturated NaCl solution containing 1% of tartaric acid (10 cc. sat. NaCl + 0.1 Gm. tartaric acid) and the ether discarded. The washings are added to the aqueous solution in the second funnel, the solution then made ammoniacal to litmus, saturated with NaCl, and extracted with four 50-cc. portions of washed ether. The ethers are combined in a 250-cc. separator and filtered through purified cotton into a 400-cc. beaker. The separator is rinsed with several portions of washed ether. The combined filtered ethers are then evaporated on a steam bath with a gentle current of air to about 20 to 25 cc. It is then transferred to a 125-cc. separator (the separator having been marked for a 35-cc. volume) with small portions of ether and made to 35 cc. with ether. The beaker is then rinsed with 10, 10, and 5 cc. of weak ammonia water (one drop 10% NH_3 in 200 cc. of distilled water), the rinsings being added to the separator containing the ether. The mixture is shaken fairly vigorously for two minutes (this time used for all subsequent shakings) and the aqueous layer withdrawn into a second separator containing 35 cc. of U. S. P. ether (not washed) and shaken. The water solution is then withdrawn into a third separator containing 35 cc. of U. S. P. ether, again shaken, and finally withdrawn into a 250-cc. separator. This whole process is repeated with six successive 10-cc. portions of the weak ammonia water. (If only the colorimetric assay is desired, the combined aqueous extracts (85 cc.) are freed from ether, made to 100 cc., and the colorimetric determination made on this solution.) About 25 to 30 Gm. of sodium chloride and 10 cc. of chloroform are added to the combined aqueous extracts. The mixture is shaken and the chloroform filtered through purified cotton into a 100-cc. beaker. The aqueous solution is extracted with five more 10-cc. portions of chloroform. The combined filtered chloroform extracts are then evaporated on a steam bath, with a current of air, just to dryness. (Do not allow to remain on the bath dry for any length of time.) The residue is dissolved in 0.01% tartaric acid, with the aid of a rubber-tipped rod and made up to volume, with 0.01% tartaric acid, generally to 50 or 100 cc. depending on the amount of ergonovine present. The solution is then assayed colorimetrically and biologically. The amount of ergonovine in this volume represents that obtained from 8.1 Gm. of drug.

For the colorimetric estimation the following procedure is recommended: Four cubic centimeters of the ergot solution is mixed with 8 cc. of the ergot reagent (0.125 Gm. *p*-dimethylaminobenzaldehyde + 0.1 cc. of 5% solution $FeCl_3$ + 65% *v/v* H_2SO_4 to make 100 cc.) in a small glass-stoppered container (a 25- or 50-cc. volumetric flask is suitable). The mixture is allowed to stand thirty minutes and the color read in a suitable photometer using a filter of about 550 or 560 mμ. A standard curve, of course, should be run on the instrument using pure ergotoxine ethanesulfonate and ergonovine maleate.

Fluidextract of Ergot.—Twenty cubic centimeters of the fluidextract is accurately measured into a 125-cc. separator, 5 cc. of 10% NaOH is

added, the mixture saturated with NaCl, and extracted with four successive 50-cc. portions of U. S. P. ether. The ether extracts are combined in a beaker and filtered through a funnel containing a pledget of cotton, into a 250-cc. separator. The beaker and funnel are rinsed with small portions of ether which is combined with the main ether solution. The ether solution at this point should be perfectly clear and free from any brown globules of the fluidextract. The ether solution is then extracted with 10 cc. and then with six successive 5-cc. portions of 1% tartaric acid. The tartaric acid extracts are combined in a small glass evaporating dish and concentrated to a volume of about 25 cc., using a gentle air blast and a temperature of about 40 to 50°. After cooling to room temperature the liquid is transferred from the dish to a 50-cc. volumetric flask, using water and made to the mark. The assay is completed from this point by the same method used above for ergot or defatted ergot, starting with the third paragraph, reading "A 5-cc. aliquot is pipetted . . . ."

Method VI, *submitted by Ralph G. Smith*

The following method is essentially that of Powell, Reagan, Stevens, and Swanson (4) with certain modifications which are believed to result in a more complete yield of total alkaloids.

I. Ergot or Defatted Ergot.—*Total Alkaloids:* Place 15 Gm. of powdered ergot in a 250-cc. flask. Add 147 cc. of reagent acetone and 3 cc. of a 10% solution of ammonia. Stopper the flask and shake it in a mechanical shaker for one hour. Filter and transfer 100 cc. of filtrate, which represents 10 Gm. of the powdered ergot, to an evaporating dish. Evaporate it at room temperature under an air current to approximately 20 cc. Transfer the solution to a 125-cc. separatory funnel. Add approximately 3 volumes of U. S. P. ether, using it to wash the evaporating dish. This acetone-ether mixture will form a layer over water. Make acid with 0.2 cc. of 20% tartaric acid solution. Extract with four 10-cc. samples of 1% tartaric acid solution, in each case drawing off the aqueous solution into a small round-bottomed flask. From the combined samples remove traces of ether and acetone by evacuation at approximately 40°. Transfer the solution to a 50-cc. volumetric flask, washing with small volumes of 1% tartaric acid solution to make to volume. Remove a 5-cc. aliquot (representing 1 Gm. of ergot) and dilute it to 50 cc. or other suitable volume for colorimetric estimation (see Method V) of total alkaloids to be expressed as ergotoxine ethanesulfonate.

Ergonovine: The pipette used for removal of the 5-cc. aliquot of total alkaloids is rinsed with about 2 cc. of water and the rinsing returned to the 45 cc. of 1% tartaric acid solution which represents 9 Gm. of ergot. Add 10% ammonia water to produce a *p*H of approximately 8. Allow to stand for ten minutes testing the *p*H at intervals and adding more ammonia if necessary to maintain a *p*H of 8. Finally make up to 50 cc. volume with distilled water and filter the mixture. Pipette a 45-cc. aliquot of the filtrate (representing 8.1 Gm. of ergot) into a separatory funnel. Shake this successively with two 50-cc. portions of carbon tetrachloride (ten minutes for each shaking) to remove the last traces of the ergotoxine-like alkaloids. Discard the carbon tetrachloride fractions and remove all traces of the latter from the aqueous fraction by reduced pressure at 40° in a round-bottomed flask. Transfer the aqueous fraction to a 125-cc. separatory funnel, saturate it with sodium chloride and shake it with five successive 50-cc. samples of ether. Evaporate the combined ether solutions to dryness with an air current and dissolve the residue in water (brought to a *p*H of approximately 4 with tartaric acid). The final volume is made up to 16.2 cc., i.e., 1 cc. represents 0.5 Gm. of ergot. The solution is then assayed colorimetrically and biologically for ergonovine.

II. Fluidextract of Ergot.—Place 12 cc. of the fluidextract of ergot in a 250-cc. flask. Add 5 cc. of 10% ammonia and 103 cc. of reagent acetone. Shake it in a mechanical shaker for one hour and filter. Evaporate 100 cc. of the filtrate, which represents 10 cc. of the fluidextract, under a current of air to dryness. Remove the residue to a separatory funnel, by dissolving in 25 cc. of distilled water. Saturate the solution with sodium chloride and make alkaline to a *p*H of 8 with ammonia water. Shake the solution with three separate 50-cc. portions of ether (ten minutes for each shaking), washing the container which contained the residue with the first portion of ether. Evaporate the combined ether solutions under a current of air to approximately 50 cc. Continue as with the method for ergot beginning with: "Make acid with 0.2 cc. of 20% tartaric acid solution."

The three samples of powdered ergot and three fluidextracts prepared from them by the method described in U. S. P. XII, along with the U. S. P. Reference Standards of Ergotoxine Ethanesulfonate and Ergonovine Maleate, were supplied by the office of the United States Pharmacopœia in April, 1945. It may be noted that the one sample of ergot (290643-A) was a defatted preparation while the other two samples were not defatted. The methods were applied directly to the two latter samples without defatting.

The collaborators in the assays were the same as those in the previous study with the exception that a report was not made by J. Allen Reese. Since this study entailed several weeks of work all reports were not submitted until late in the summer of 1945.

The results are recorded in Table II. Since there were five collaborators reporting on three samples of ergot, fifteen comparisons between the two methods are available for each type of determination except in those instances where a complete re-

TABLE II.—SECOND COLLABORATIVE ASSAY: SUMMARY OF REPORTS

Total Alkaloids. Colorimetric method. Results expressed as mg. of ergotoxine ethanesulfonate per Gm. of Ergot or per ml. of Fluidextract.

Ergonovine. Results expressed as mg. of ergonovine maleate per Gm. of Ergot or per cc. of Fluidextract.

Report No.	Powder			Fluid Extract		
	Total Alkaloids	Ergonovine Colorimetric	Ergonovine Bioassay	Total Alkaloids	Ergonovine Colorimetric	Ergonovine Bioassay
	Defatted Ergot 290643-A—Method V					
1	3.27	0.244	0.235	1.80	0.193	0.181
2	3.39	0.23	0.16	1.94	0.20	0.14
3	3.30	0.235	0.205	1.59	0.213	0.18
4	3.95	0.207	0.179	1.86	0.158	0.150
5	2.55	0.167	0.162	2.52	0.154	0.18
Av. ± S. D.	3.29 ± 0.50	0.217 ± 0.031	0.188 ± 0.031	1.94 ± 0.34	0.184 ± 0.027	0.166 ± 0.020
	Defatted Ergot 290643-A—Method VI					
1	3.16	0.291	0.178	1.20	0.291	0.151
2	3.35	0.27	0.19	2.01	0.24	0.14
3	3.00	0.227	0.187	1.69	0.210	0.19
4	3.11	0.251	0.195	1.28	0.315	0.198
5	2.00	0.216	0.175	0.81	0.144	0.158
Av. ± S. D.	2.92 ± 0.53	0.251 ± 0.031	0.185 ± 0.009	1.40 ± 0.46	0.240 ± 0.067	0.167 ± 0.026
	Ergot A 45—Method V					
1	2.83	0.246	0.231	1.87	0.157	0.130
2	3.10	0.24	0.20	2.01	0.20	0.15
3	2.77	0.254	0.22	2.00	0.204	0.18
4	3.21	0.227	0.131	1.67	0.184	0.162
5	2.20	0.167	0.150	2.80	0.167	0.195
Av. ± S. D.	2.82 ± 0.39	0.227 ± 0.036	0.186 ± 0.045	2.07 ± 0.43	0.182 ± 0.020	0.163 ± 0.025
	Ergot A 45—Method VI					
1	2.75	0.260	0.167	1.16	0.274	0.132
2	2.90	0.25	0.20	1.97	0.26	0.20
3	2.45	0.245	0.238	1.75	0.225	0.19
4	3.20	0.238	0.141	...	...	...
5	1.92	0.184	0.161	1.04	0.156	0.160
Av. ± S. D.	2.64 ± 0.49	0.235 ± 0.029	0.182 ± 0.038	1.48 ± 0.45	0.229 ± 0.043	0.170 ± 0.036
	Ergot B 45—Method V					
1	2.59	0.250	0.236	1.85	0.148	0.117
2	2.81	0.21	0.17	2.20	0.20	0.18
3	2.80	0.300	0.27	1.80	0.298	0.25
4	3.12	0.218	0.217	2.45	0.174	0.153
5	1.92	0.123	0.077	2.75	0.154	0.179
Av. ± S. D.	2.65 ± 0.45	0.220 ± 0.065	0.194 ± 0.075	2.21 ± 0.40	0.195 ± 0.061	0.176 ± 0.049
	Ergot B 45—Method VI					
1	2.63	0.246	0.154	1.51	0.278	0.120
2	2.81	0.23	0.16	2.12	0.24	0.19
3	2.50	0.280	2.80	1.60	0.317	0.24
4	2.60	0.118	0.118	...	...	...
5	1.90	0.172	0.153	1.08	0.152	0.130
Av. ± S. D.	2.49 ± 0.36	0.209 ± 0.065	0.173 ± 0.062	1.58 ± 0.42	0.247 ± 0.070	0.170 ± 0.056

port was not submitted. The following observations may be made:

1. Method V gave higher results for total alkaloids in 13 of 15 comparisons (one equal) on the powders and in 11 of 13 comparisons on the fluidextracts. This indicates a more complete extraction of total alkaloids by Method V. In the 13 instances of a higher yield by Method V for the powders, 5 were less than 5% higher and 10 were less than 15% higher than the yield by Method VI. The differences with the fluidextracts were greater. In the 11 instances of a higher yield by Method V only 2 were less than 5% higher and only 4 were less than 15% higher than the yield by Method VI.

2. Method V gave lower results for the colorimetric assay of ergonovine in 10 of 15 comparisons on the powders and in 9 of 13 comparisons on the fluidextracts. This would be in keeping with a more complete removal of ergometrinine from the water-soluble fraction by the Grove procedure in the majority of instances.

3. Method V gave lower results for the bioassay of ergonovine in 8 of 15 (one equal) comparisons on the powders and in 7 of 13 (one equal) on the fluidextracts.

4. In a comparison of the colorimetric and bioassay results for ergonovine the table shows that the average value for each sample is higher for the

former than for the latter. In a comparison of 30 individual results on the powders the same holds true in 27 instances, three being equal. For 28 individual results on the fluidextracts the same holds true for 23 results while 5 results—all from one laboratory—show higher values for the bioassay. The percentage differences between the colorimetric and bioassay values were calculated in these 58 possible comparisons. On consideration of those percentage differences it was found that the colorimetric value exceeded the bioassay value as follows: (*a*) Powders, Method V, 15 comparisons—av. 23 ± S. D. 21%; (*b*) Powders, Method VI, 15 comparisons—av. 31 ± S. D. 23%; (*c*) Fluidextracts, Method V, 15 comparisons—av. 12 ± S. D. 17%; (*d*) Fluidextracts, Method VI, 13 comparisons—av. 36 ± S. D. 42%. Thus both methods in the great majority of instances yield higher values for ergonovine by the colorimetric procedure and the relationship between the colorimetric and bioassay values show considerable variation, more so by Method VI than by Method V.

Comments on the methods and results were submitted with the assay reports and there was further discussion in this regard at a meeting in Atlantic City in March, 1946, attended by three of the collaborators and Dr. M. W. Green representing the National Formulary. These comments and discussions are not reported here in detail. Those pertaining to results have been covered above in the digest of Table II. Pertinent remarks regarding the technique of the methods are as follows: (*a*) In Method VI, after evaporation of the acetone extract of the fluidextract to dryness, considerable trouble with emulsification was experienced in the subsequent shakings with ether. It was suggested that this could be avoided by evaporation of the acetone extract to approximately 10-cc. volume rather than to dryness. (*b*) In both methods the filtration after precipitation of ergotoxine with ammonia could be speeded by filtration through a fine glass filter under suction. (*c*) The dosage of estrone advised in the bioassay method of Vos (8) often resulted in uteri unsatisfactory from the standpoint of too much spontaneous activity. One collaborator found that two doses of 150 mg. of estrone on successive days proved to be satisfactory.

At this point in the study it was necessary to reach a decision on three questions. (*a*) Could a recommendation of a method for the assay of ergot and its fluidextract be made on the basis of the work to date? (*b*) If a method were recommended should it be Method V or Method VI? (*c*) If a method were recommended should it include a bioassay for ergonovine or would the colorimetric assays for total alkaloids and for ergonovine be sufficient? Opinion was divided on all questions so that the decisions were compromises although no strong objection was raised to the actions which were taken.

It was decided that a recommendation of a method be made at this time. It was the opinion of some collaborators that such could be done on the basis of the results already obtained. The action was further forced to some extent by the fact that certain members of the group did not find it feasible to conduct further series of assays on account of the pressure of other duties.

It was decided to recommend Method VI as the extraction procedure for the assay. It is true that Method V usually yielded somewhat higher results for total alkaloids, indicating more complete extraction, and somewhat lower colorimetric results for ergonovine with less variation from the bioassay results. In spite of this fact the differences in the results of the two methods were not considered to be sufficiently great to outweigh the advantage of Method VI, as a practical method of assay, from the standpoint of the time and labor involved.

It was decided to recommend that ergonovine should be assayed only colorimetrically. Although the colorimetric results were almost always higher than the bioassay results there was no evidence for a greater variation in the colorimetric than in bioassay results from different laboratories. For one ergot and fluidextract assayed by Method VI the colorimetric results showed greater variation, while for the other two ergots and fluidextracts the bioassay results showed greater variation. Consequently, it was believed that a colorimetric assay would yield a sufficiently accurate estimate of the ergonovine content.

Method VI, involving the colorimetric assay of total alkaloids and of ergonovine, was accordingly recommended to the National Formulary for further trial under its auspices. In the description of the method which was submitted there were minor modifications in wording and the two following changes from that described above:

(*a*) At the end of the procedure for the extraction of ergonovine—"The final volume is made up to 16.2 cc. . . . assayed colorimetrically and biologically for ergonovine." was changed to "The final volume is made up to 50 cc. or other suitable volume (representing 8.1 Gm. of ergot) for colorimetric estimation . . . "

(*b*) In the assay of fluidextract of ergot—"Evaporate 100 cc. of the filtrate . . . to dryness." was changed to "Evaporate 100 cc. of the filtrate . . . to a volume of approximately 10 cc."

More recently, Swanson (9) has suggested a modification in Method VI which, according to his experience, is an improvement. In the assay of ergonovine, instead of precipitating the ergotoxine-like alkaloids from the extract of total alkaloids by the addition of ammonia water, the precipitation is made by alkalinizing with sodium bicarbonate to produce a *p*H of approximately 8. The superiority of sodium bicarbonate arises from the fact that with ammonia the *p*H changes too rapidly by evaporation.

The Sub-Group which conducted this study took no formal action on requirements of potency for ergot or the fluidextract of ergot.

SUMMARY

A report is made on a collaborative study of the assay of ergot and the fluidextract of ergot by a Subgroup of the Subcommittee on Biological Assays of the United States Pharmacopœia. The results of the study were later made available to the Committee on the National Formulary. Two collaborative assays involving a comparison of various methods for the extraction of ergonovine are described in detail. A method was finally recommended for the consideration of the Committee on the National Formulary in which ergot and the fluidextract of ergot are assayed colorimetrically for total alkaloids and colorimetrically for ergonovine.

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Pharmacological Action of Senecio Riddellii*

By IRENE ROSENFELD and O. A. BEATH†

The administration of the crude alkaloid of *Senecio riddellii* in mice produced acute intoxication followed by remission, recurrence of symptoms, and death. The toxic dose was determined by intraperitoneal and oral administration of the alkaloid. The feeding of plant produced typical symptoms of chronic poisoning similar to that observed in cattle. The alkaloid-free resinous residue produced vascular damage in the intestinal tract with subsequent development of gangrene. The injection of the crude alkaloid of *Senecio riddellii* reduced the vitamin A content of the liver.

THE GENUS *Senecio* comprises over 1250 different species, and it is the largest genus belonging to the order *Compositae*. Many species have been used for the treatment of diseases. The species most generally described to have medical value were *S. vulgaris* and *S. jacobaea*. The plant and the fluidextract of *S. aureus* was official in the National Formulary as late as the fifth edition.

Certain species are poisonous for cattle and horses and were described by various investigators in New Zealand, South Africa, Canada, and the United States. The toxicity and the physiological action of the plants are due to the presence of an alkaloid. The alkaloid of *Senecio* is unique in its action in that it stimulates the central nervous system and at the same time damages the parenchymatous organs. A constant pathological lesion described by all investigators in chronic poisoning was cirrhosis of the liver.

Investigations of the toxic principles present in species of *Senecio* has resulted in the isolation of a large number of alkaloids. More recently Manske (1–3) published a series of papers on the isolation of *Senecio* alkaloids. Several of the alkaloids have been subjected to pharmacological investigation. Cushny (4) was the first investigator who studied the pharmacological action of senecifoline. He divided the symptoms of the alkaloid intoxication into acute, subacute and chronic. He concluded that the acute symptoms were due to stimulating action of the alkaloid on the upper part of the central

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nervous axis. He was unable to produce true hepatic cirrhosis but he explained that this was due to shorter duration of intoxication in the smaller laboratory animals. Harris, Anderson, and Chen (5) studied the action of riddelline, an alkaloid of *Senecio riddellii*, and reported that the alkaloid produced the characteristic liver lesion in experimental animals. Their investigations were limited to the study of the acute effect of the alkaloid.

Our investigations were undertaken to observe the acute, subacute, and chronic symptoms in experimental animals; to study the comparative toxic action of the dried plant, crude alkaloid and the alkaloidal-free resinous residue of *Senecio riddellii;* and to study the effect of the alkaloid upon the vitamin A content of the liver.

EXPERIMENTAL

The crude alkaloid was prepared essentially by the method of Manske (2). Single, graded doses were administered intraperitoneally and orally to white mice weighing from 20 to 25 Gm.

The air-dried, ground plant was added in 10, 20, 30, 40, and 50% quantities to the ground stock feed, mixed thoroughly and bound together by the addition of egg and milk.

The resinous alkaloid-free residue was obtained after the alkaloid extraction, composed of all the ingredients of the plants except the alkaloid. This alkaloid-free residue was added in 1, 5, 10, 20, and 30% concentrations to the stock feed, mixed by passing through a food chopper several times and also bound together by the addition of egg and milk.

These diets were fed to groups of six mice until death. Control groups receiving stock diet were used. After the death of the last experimental animal, the controls were killed and examined grossly and microscopically. There were no pathological changes present in the control groups.

The crude alkaloid of *Senecio riddellii* was injected intraperitoneally in white mice weighing from 20 to 25 Gm. for vitamin A determinations in the liver. Mice were autopsied soon after death. The livers of the mice were removed immediately and determinations for vitamin A were carried out according to the method of Lewis, Bodansky, Falk, and McGuire (6).

RESULTS

Symptoms of acute toxicity were observed in groups of mice which were injected intraperitoneally and in those in which the crude alkaloid was given orally. In both groups the higher doses caused the most pronounced symptoms of hyperactivity, increased respiration, and clonic convulsions resembling cocaine or other convulsive poisons. After recovery from the primary symptoms, the animals appeared normal from several hours to several days. After this time they became listless, anoretic, the fur seemed to be ruffled, and the animals sat huddled, the body swaying from side to side. Before death the respiration became labored, the animals had numerous convulsions, and death occurred within a few minutes after the second attack. Ten to twenty per cent loss in weight occurred in mice which lived for a few days. The results of the intraperitoneal and oral administration of the crude alkaloid are given in Tables I and II. The minimum lethal dose of the crude alkaloid by the intraperitoneal injection was 325 mg., while in the oral group it was 400 mg. per Kg. Duration of life after the intraperitoneal injection of 500 mg. per Kg. of crude alkaloid was fifteen hours whereas the same quantity of crude alkaloid *per os* was fatal only in two and one-half days. If 1000 mg. per Kg. of the crude alkaloid was injected intraperitoneally, the animals died within five minutes. However, the same quantity caused death in twelve hours by oral feeding. This difference may be explained by the slower absorption of the alkaloid from the intestinal tract. Each mouse

TABLE I.—TOXICITY OF SENECIO RIDDELLII CRUDE ALKALOID INJECTED INTRAPERITONEALLY

Dose, Mg./Kg.	No. of Animals	Deaths, %	Survivals,[a] %	Av. Death Time, Hr.
60	4	...	100	..
80	4	...	100	..
100	8	...	100	..
150	4	...	100	..
200	4	25	75	96
250	4	25	75	96
300	8	37.5	62.5	48
325	8	50	50	72
350	4	75	25	24
400	4	100	...	23
450	4	100	...	19
500	4	100	...	15
Control[b]	4	...	...	..

[a] Killed 14 days after injection.
[b] Killed at the end of the experiment.

TABLE II.—TOXICITY OF SENECIO RIDDELLII CRUDE ALKALOID PER OS

Dose, Mg./Kg.	No. of Animals	Deaths, %	Survivals,[a] %	Av. Death Time, Days
300	4	25.0	75.0	7.0
350	4	25.0	75.0	5.0
400	8	50.0	50.0	3.0
500	8	62.5	37.5	2.5
600	8	62.5	37.5	3.0
700	4	75.0	25.0	2.0
800	4	100.0	..	1.0
900	4	100.0	..	1.5
1000	4	100.0	..	0.5
Control	4			

[a] Killed 14 days after injection.
[b] Killed at the end of the experiment.

after death was autopsied and examined for pathological changes. Gross and microscopic pathology observed in the alkaloid injected group was capillary damage followed by liver necrosis. The pathological changes produced by the *Senecio riddellii* alkaloid were reported elsewhere (7).

The mice fed with various quantities of plant showed the same symptoms of chronic *Senecio* poisoning as observed in cattle and horses by Gilruth (8). Table III gives the results obtained. The duration of life of animals receiving 30, 40, and 50% plant in their diets was fifty-five, fifty-three, and fifty-four days, respectively. The mice were cachectic and anoretic before death. At autopsy there was nodular and diffuse hepatic cirrhosis present (Figs. 1 and 2). Other organs showed similar chronic degenerative changes. In the 10% and 20% groups

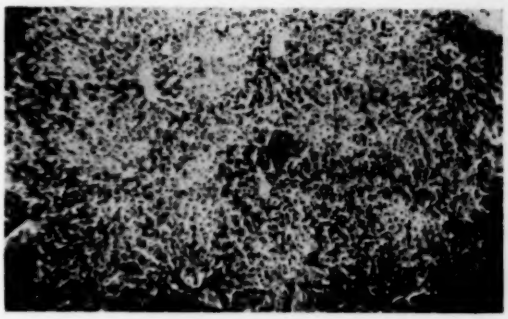

Fig. 2.—Diffuse cirrhosis of the liver.

The liver cells were compressed by the newly formed fine fibrous tissue. The fibrous tissue was distributed diffusely between the liver parencyma.

TABLE III.—TOXICITY OF SENECIO RIDDELLII

No. of Animals	Plant in the Diet, %	Av. weights, Gm. Beginning of Experiment	Time of Death	Total Amount of Plant, Gm.	Av. Death Time, Days
6	10	26.1	25.9 (13.1)[a]	68	91
6	20	25.0	26.1 (17)[a]	84	84
6	30	24.7	16.2	120	55
6	40	23.8	15.2	132	53
6	50	26.3	12.2	165	54
6	Control[b]	25.6	29.5	...	..

[a] Weights before edema developed.
[b] Killed at the end of the experiment.

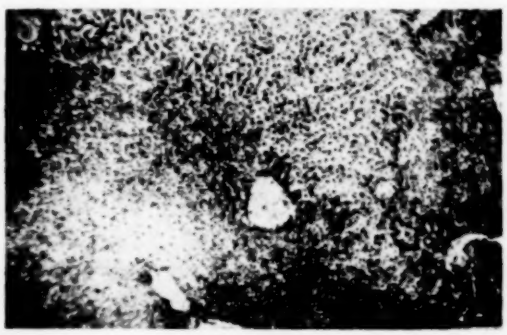

Fig. 1.—Nodular cirrhosis of the liver.

The normal liver cells around the central vein were compressed and surrounded by fibrous tissue. Several liver lobules appeared to be grouped around the central vein. The nodules were composed largely of cells which were irregular in size and arrangement.

extensive edema developed a few days before death, which fact is evident from the changes in weight shown in Table III.

Table IV gives the results obtained with the alkaloidal-free resinous residue. Duration of life was shorter in this group than in those fed with the plant. At autopsy all animals on the diet containing the plant residue showed gangrene of the digestive tract. The other organs appeared normal. There was no fibrosis or necrosis present in the liver, which was observed in all cases of *Senecio* alkaloid poisoning. Intestinal disturbances observed by Gilruth (8), Cushny (4) and other investigators may not be alkaloidal in nature but they may be due to some irritating substances present in the alkaloid-free residue. Whether the residue contains a corrosive substance which causes vascular damage in the intestinal tract or whether this damage was due to the accumulation and irritating action of the resinous material, we cannot state definitely at the present time. A fractional chemical study of the alkaloidal-free resinous residue must be made before the causative agent of the observed pathological change can be determined.

TABLE IV.—EFFECTS OF SENECIO RIDDELLII ALKALOIDAL RESIDUE

No. of Animals	Plant Residue in the Diet, %	Av. weights, Gm. Beginning of Experiment	Time of Death	Total Amount of Residue, Gm.	Av. Death Time, Days
6	1	22.7	17.2	6.4	57
6	5	24.2	18.5	29.5	55
6	10	22.2	16.2	32	31
6	20	22.6	15.6	40	22
6	30	23.2	16.2	42	15
6	Control[a]	22.8	25.8	..	..

[a] Killed at the end of the experiment.

Many observations suggest that toxic substances which injure the reticulo-endothelial system of the liver may alter the metabolism of vitamin A. Goer-

ner (9) reported the reduction of the vitamin A content of liver mitochondria after intraperitoneal injection of dibenzanthracene, while *p*-aminoazobenzene was without effect. Reduction of hepatic vitamin A by the intraperitoneal injection of colloidal dibenzanthracene was reported by Baumann, Foster, and Lavik (10). They found that methylcholanthrene, benzpyrene, and 1,2-benzanthracene likewise reduced hepatic vitamin A, but all these were less effective than dibenzanthracene.

TABLE V.—RELATION OF DOSAGE AND LIVER INJURY TO THE VITAMIN A IN THE LIVER

Dose, Mg./Kg.	No. of Mice	Duration of Life	Liver Injury[a]	Vitamin A, Mμ/Gm.
325	4	14 days, killed	Normal	30.8
325	2	38 hr.	+	23.7
325	2	48 hr.	+++	13.7
350	4	36 hr.	Hemorrhage and congestion	26.4
350	5	36 hr.	++	17.3
400	4	35 hr.	+++	10.2
400	4	30 hr.	+++	17.1
450	5	22 hr.	+, Hemorrhage and congestion	19.7
450	1	5 min.	Normal	28.7
500	3	13 hr.	+	14.3
500	5	22 hr.	++	12.3
Control	5	..	.....	34.5

[a] +Focal necrosis. ++ Necrosis of one lobe. +++Necrosis of 2 or more lobes.

From Table V it is evident that there is a definite relation between the vitamin A content of the liver, the extent of liver injury, or the amount of alkaloid injected. Liver which showed only acute congestion and hemorrhage contained normal amount of vitamin A. There was a decrease in the amount of vitamin A in the liver in which necrosis of one or more lobes was present. The injection of 500 mg. per Kg. of alkaloid reduced the vitamin A content of the liver over 50%, suggesting that action of the alkaloid may be directly responsible for the lowered vitamin A content in the liver.

CONCLUSIONS

Crude alkaloid of *Senecio riddellii* was administered intraperitoneally and *per os* in single, graded doses to mice. The minimum lethal dose was determined by both methods. The symptoms in both groups were acute intoxication, followed by remission, recurrence of symptoms, and death.

Feeding of the plant produced chronic symptoms, that is, cachexia, anorexia, edema, and nodular cirrhosis of the liver similar to that observed in cattle and horses.

The alkaloidal-free resinous residue damaged the intestinal tract, with the subsequent development of gangrene. Whether the injury was due to a corrosive toxin present in the residue or whether the injury was mechanical in nature cannot be determined from the present investigation.

The injection of crude alkaloid of *Senecio riddellii* produced acute hepatitis and reduced the amount of vitamin A in the liver. Higher concentrations of the alkaloid had similar effect on the vitamin A content of the liver.

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The Chronic Toxicity of Hydroxyethyl Cellulose for Rats*

By HENRY F. SMYTH, JR., CHARLES P. CARPENTER, and CARROL S. WEIL

Indirect evidence, based on feeding experiments to rats during a two-year period, indicates that hydroxyethyl cellulose is neither absorbed or hydrolyzed in the gastrointestinal tract.

VARIOUS HYDROPHILIC cellulose derivatives are technically suitable for oil-resistant surface coatings, binders, adhesives, thickeners for aqueous solutions, and the like. Often these applications involve contact with foods, or use in cosmetics and pharmaceuticals, both of which require physiologically inert materials. The large molecular dimensions of cellulose ethers suggest that they may not be absorbed from the digestive tract, and their chemical stability is indirect evidence that no diffusible hydrolysis products are formed as they pass through the body. Nevertheless, experimental evidence upon the question of low toxicity is desirable before any new chemical is applied in ways which might endanger health, no matter how strong an *a priori* case may be drawn for the safety of the application.

Deichmann and Witherup (1) administered methyl and ethyl cellulose to rats in the food and drinking water over a period of eight months. The consumption of daily doses of 0.44 and 0.18 Gm., respectively, by each rat failed to produce any evidence of injury. Machle, Heyroth, and Witherup (2) could find no chemical change in methyl cellulose resulting from its passage through the human digestive tract and no increase in the excretion of methanol resulting from its ingestion. Hodge (3) fed large amounts of cellulose acetate phthalate to rats and dogs in their diet for one year without causing any injuries. Brown and Houghton (4) state that the sodium salt of carboxymethyl cellulose is harmless in animal feeding but they furnish no details. Weile (5) fed large amounts of the same salt to rats and dogs for two months with the same absence of injury. Rowe and associates (6) could find no injury caused by cellulose glycolic acid after prolonged feeding to animals. The foregoing negative results confirm deductions based on molecular size and chemical stability.

A newly available compound, water-soluble hydroxyethyl cellulose, has been given the trade-mark name of "Cellosize." The only publication bearing upon the toxicity of this material is that of Hueper (7), who studied cardiovascular pathology following its intravenous injection in dogs. As many as 55 injections of 2.5 Gm. each were made in a small number of dogs during eleven weeks without producing injury other than temporary alterations in the physical properties of the blood, hemoglobin, blood cytology, and blood vessel walls. These changes were presumably caused by the physical properties of the material injected.

Increasing applications for "Cellosize" brand of hydroxyethyl cellulose prompted a study here of its oral toxicity. The viscosity of dispersions in water limited the single oral dose which could be administered to albino rats to 8.7 Gm./Kg. in the form of a 10.9 per cent aqueous dispersion. This dosage killed none of 10 adolescent male rats within a fourteen-day observation period, did not interfere with their appetite or growth, and produced no micropathology.

Accordingly a program was undertaken calling for daily doses of the material in the diet over a period of two years. The sample was a commercial product, manufactured in October, 1944 in the form of a spray-dried, freely flowing white powder, designated "Cellosize" WPLM. It contained 11.43 per cent moisture, and 2.43 per cent ash on a dry basis. A 5 per cent solution in water yielded a *p*H of 6.45, and the viscosity at 20° of the same solution was 66.1 centipoises. On the basis of these specifications it is calculated that the sample contained 86.43 per cent of hydroxyethyl cellulose, the balance being chiefly water. The various quantities men-

* Received April 18, 1947, from Mellon Institute, Pittsburgh, Pa.

tioned in the following pages are in terms of the sample as it was received, not of anhydrous chemical.

There are also available commercially alkali-soluble types of hydroxyethyl cellulose which differ in properties and applications from the water-soluble type. Only water-soluble "Cellosize" hydroxyethyl cellulose was investigated in this study.

METHODS

The cellulose ether was administered to rats as one component of a dry diet consisting of 1000 Gm. ground wheat, 500 Gm. dried whole milk, and 20 Gm. of iodized salt. After eighteen months wheat became unobtainable, and a shift was made to Purina Laboratory Chow Meal. Three dosage levels were fed, consisting of 5, 1, and 0.2% of cellulose ether added to the basic diet, in addition to a control diet containing no added ingredient. The mixtures were available continually in modified McCollum food cups which allow only a negligible amount of spillage. For the sake of variety the animals were furnished supplements of oranges, kale, carrots, liver, and marrow bones.

Wistar strain albino rats one month old were observed for two weeks so as to discard obviously weak animals. The selected rats were known as the parental generation. Each dosage level was given to 16 males and 16 females, housed 2 of each sex in a wire bottom cage. Offspring were kept until at least 10 of each sex representing 10 litters from each dosage level had attained a weight of 40 Gm. These rats of the F_1 generation were maintained on the diets until the end of the study. A total of 200 rats was observed.

Running records were kept upon body weight, diet consumed, and litters produced. Blood counts were performed several times on several rats at each dosage level. All animals dying were autopsied to determine cause of death, and, when autolysis had not set in, tissues were prepared and studied microscopically. A particular search was made for neoplastic growths. After two years on the diets the animals were sacrificed for examination and pathological study.

RESULTS

The levels of 5, 1, and 0.2% "Cellosize" in the diet resulted in mean dosages of 2.31, 0.41, and 0.09 Gm./Kg./day, or about one-third these latter values in terms of grams per rat per day. The rats ate one-tenth more of the 5% diet than of the lower levels or the control diet, partly, no doubt, to compensate for the undigested component. Their feces were almost white and bulkier than normal because of the content of white hydrophilic cellulose ether. Because of the low magnitude of the effect and absence of supporting evidence one would be rash to say that the increased food intake was due to better health from improved fecal consistency. The study lasted for the major part of the normal life span of the rats, and in the course of this time 48% of the animals died. Every death was caused by a recognizable factor distinct from the doses, and the fatalities were evenly distributed between the four dosage groups.

The other criteria considered in the determination of toxic response to the chemical were frequency of extraneous infections, life span, rate of increase in body length and weight, ratio of weight to length as an index of fatness, kidney and liver weights, frequency and size of litters, blood cytology, occurrence of neoplasms, and micropathology. The organs examined were adrenal, small intestine, kidney, liver, lung, pancreas, spleen, and testis. All numerical data were examined by the familiar "t" test, with the fiducial point at the significant level of 1 in 20 for "p." Except for increased food intake mentioned above none of the criteria revealed any relation between the dosage and the response, and hence the three dosages were devoid of harmful effect.

A small number of rats in each of the four groups developed tooth anomalies. Their incisors were not worn off by the opposing teeth, and, continuing to grow unchecked, entirely prevented food intake and resulted in starvation. This result is attributed to the absence of abrasion because of the soft powdered diet. The uniform distribution of the condition among control and treated animals alike shows that it was not caused by the presence of cellulose ether in the diet.

SUMMARY

A parental and an F_1 generation of Wistar strain albino rats totalling 52 for each dosage level and control were maintained for two years upon diets containing added "Cellosize" hydroxyethyl cellulose WPLM in concentrations of 5, 1, and 0.2 per cent. The resulting mean dosages were, respectively, 2.31, 0.41, and 0.09 Gm./Kg./day. The dosages were devoid of effect, except that the food intake of rats eating the 5 per cent diet was one-tenth greater than that of animals in the other 3 groups.

The results are indirect evidence that the compound is neither absorbed nor hydrolyzed in the gastrointestinal tract.

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The Demonstration in Amorphous Penicillin of a Non-Penicillin Factor Which Enhances Its Protective Action*

By HENRY WELCH, W. A. RANDALL, and C. W. PRICE

The presence of a protective enhancement factor in amorphous penicillin has been demonstrated in a large proportion of penicillin samples examined. A method is described for determining the presence of this factor which enhances the activity of penicillin but is itself devoid of *in vivo* activity.

DURING THE PAST two years there has appeared in the literature information indicating that in certain lots of amorphous penicillin there are substances of possible therapeutic importance which do not appear in the crystalline product. In July, 1944 Cornman (1) stated that there was present in various commercial penicillin preparations an agent which he found exerted a selective lethal effect upon rat and mouse sarcoma cells growing with normal cells in tissue cultures. Subsequent studies, along the same lines, by Lewis (2) showed that the selective effect was not exerted by highly purified colorless penicillin, but rather that the effect was due to some substance present in the less highly purified material, along with the bacteriostatic factor. In March, 1945 Dunham and Rake (3), in reporting on the effect of penicillin G on *Treponema pallidum* showed that crystalline penicillin G had little or no effect on the motility of this organism when it was exposed for two hours *in vitro* to 8800 u./ml. while under the same conditions 2200 u./ml. of the impure penicillin employed in their studies immobilized all of the spirochetes. They stated that the substance in the amorphous penicillin could be concentrated by adsorption on alumina and recovered by elution, and that its activity was not significantly reduced when exposed to the action of penicillinase. They further showed that the immobilization produced by the amorphous penicillin was probably not due to penillic acid or to products resulting from the hydrolysis of pure penicillin. G. Smith (4) has demonstrated that penicillins G, K, and dihydro F are all relatively ineffective in inhibiting germination of seeds, while commercial penicillin may be readily shown to contain antigermination activity. He concluded that the substances present in commercial penicillin which cause inhibition of germination are represented by an indole-3-acetic acid type of compound.

At the penicillin conference held in March, 1946, Clowes (5) stated that on the basis of his studies he had reason to believe that there was something in commercial penicillin which prevented the development of marine eggs while crystalline penicillin under similar test conditions had no such effect. At the same meeting Hobby (6), in discussing the chemotherapeutic action of various penicillins on hemolytic streptococcal infections in mice, pointed out that 60 to 100 units of crude penicillin manufactured at various times from 1943 to 1946 was found to be as effective as 200 to 300 units of crystalline G penicillin. Furthermore, with a sample known to contain 30 to 40 per cent penicillin K, complete protection was obtained in mice with fewer units than would give complete protection when crystalline G or pure penicillin K was used. Hobby stated at that time that crude or partially purified penicillin was more effective than crystalline penicillin G against hemolytic streptococcal infections in mice, and that the presence of penicillin K, although it is less effective when pure than crystalline G, does not alter the protective action of crude penicillin preparations.

During the past year, the basic manufacturers of penicillin have made strenuous efforts to place crystalline material on the market. With some manufacturers this has been to the exclusion of the amorphous ma-

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terial. In view of the data available, even though somewhat tenuous, it appears questionable whether amorphous penicillin should be eliminated from the market. In the Food and Drug Administration laboratories, we are in rather a fortunate position in that we have available to us the products of each of the basic manufacturers and over the period of several years that we have been testing penicillin there has accumulated a number of vials of old lots of amorphous penicillin from various manufacturers. It occurred to us sometime ago that an attempt should be made to determine whether it was possible, in the absence of *in vitro* methods, to demonstrate by some biological method the presence or absence of this unknown factor which appeared to exist in certain lots of commercial amorphous penicillin. The present study is concerned with (*a*) the development of a method for demonstrating the presence of an enhancement factor in commercial penicillin, (*b*) the use of the method in screening various lots of penicillin of the fifteen basic manufacturers to determine the percentage of lots containing the "factor," and (*c*) miscellaneous studies dealing with stability, the effect of acid, penicillinase, heat, etc., on the "factor."

METHOD

In determining the presence of the protective enhancement "factor" in amorphous penicillin, white mice weighing 17–25 Gm. are used. Both the penicillin and the test culture (*E. typhosa* P. C. I. 412) are injected intraperitoneally thirty minutes apart. Mucin is not used. Each lot of penicillin under test is injected into 50 mice and, similarly, 50 mice each are injected with 2000, 3000, and 4000 units of crystalline sodium penicillin G as a control. In addition, 50 mice are injected with *E. typhosa* alone, to determine the lethal dose for each test. It required approximately three minutes to inject 50 mice, using five people passing the animals, one injecting and one filling syringes. In a test 500 mice are divided into groups of 50 and one such group is injected with 0.4 ml. of a six-hour broth[1] culture (calculated LD_{90}) as a control on the virulence of the organism used. Following this, the other mice are then injected consecutively in groups of 50 each three minutes apart with the penicillin under test (0.4 ml. containing the appropriate amount of penicillin). At the end of thirty minutes, the first group injected with penicillin then received a six-hour broth culture of *E. typhosa* (0.4 ml. per mouse) followed by the second group, third, etc., until all 450 mice have been injected with the culture. Deaths are recorded at the twenty-fourth and forty-eighth hour. The protective effect of each lot of amorphous penicillin is calculated by comparing the death rates obtained with those found for the different concentrations of crystalline sodium penicillin G utilized in the test. In Table I are given the results obtained on a single test using 500 mice.

It will be noted that 0.4 ml. of the six-hour broth culture of *E. typhosa* gave an LD_{90} in this test. When 2000 units of crystalline penicillin was administered for protection, 44% of the mice were saved; with 3000 units of this material, 60% of the mice were saved while 78% of the mice were saved with 4000 units. The amorphous penicillin lots 1-P-1, 62, and G403A051 all injected at the 2000 unit level saved 70%, 62%, and 84% of the mice, respectively. Thus, 2000 units of lots 1-P-1 and 62 have a protective effect equivalent to at least 3000 units of crystalline sodium penicillin G, while 2000 units of lot G403A051 has a protective effect equivalent or better than that secured with 4000 units of crystalline sodium penicillin G. Neither of the lots G504A015 and "penicillin F" contains the "factor," while in the "strontium penicillin" there may be a small amount of the "factor" present.

The effect of the "factor" on the protective action of crystalline penicillin G is shown in Table II.

TABLE I.—PROTECTIVE ACTION OF AMORPHOUS VS. CRYSTALLINE PENICILLIN VARIOUS COMMERCIAL LOTS

Penicillin Used	Penicillin Units	No. Mice	Deaths 24 Hr.	Deaths 48 Hr.	Dead, %	Unknown Factor[a]
Culture only	2000	50	43	45	90	..
Crystalline G	2000	50	22	28	56	..
Crystalline G	3000	50	15	20	40	..
Crystalline G	4000	50	6	11	22	..
Amorphous 1-P-1	2000	50	12	15	30	+
Amorphous 62	2000	50	12	19	38	+
Amorphous G403A051[b]	2000	50	7	8	16	+
Amorphous G504A015	2000	50	22	30	60	−
Strontium Penicillin	2000	50	18	24	48	−
Penicillin F	2000	50	18	28	56	−

[a] A lot was not considered to contain the "factor" unless it showed at least a 50% enhancement.
[b] Contains more than 10% penicillin "X."

[1] Federal Register, Oct. 17, 1946, p. 12128.

It will be noted that the two thousand units of crystalline penicillin sodium G saved 46% of the mice injected; 3000 units saved 58%, while 4000 units saved 78%. Lot FP-21, which showed no penicillin activity by assay (0 unit per vial) was made up to 100,000 units per vial by the addition of crystalline sodium penicillin G. Two thousand units of this mixture saved 58% of the mice injected and thus it corresponded in effectiveness to 3000 units of crystalline penicillin sodium G alone. Similarly, lot SD-5C which, on assay, showed zero units per vial, when made up to 100,000 units with crystalline sodium penicillin G, saved 70% of the mice when 2000 units were used for treatment. This mixture, therefore, was more effective at the 2000-unit level than over 3000 units of crystalline sodium penicillin G alone. The lot marked "Code 19"[2] had an assay value of 27,000 units per vial. When this lot was made up to 100,000 units with crystalline sodium penicillin G, 2000 units of this mixture protected 78% of the animals. Thus this mixture at the 2000 unit level was as effective in protecting mice against typhoid infection as 4000 units of crystalline sodium penicillin G alone. These three lots (FP-21, SD-5C, and "Code 19") all contained the protective enhancement "factor." "Code 19" obviously has considerably more of the "factor" present than the other two preparations tested. The penicillin in Lot 782H39A, previously shown to contain the "factor," was completely inactivated by clarase and then utilized to determine its protective effect against *E. typhosa*. This material (although devoid of penicillin activity) was assumed to have a potency of 100,000 units per vial and an aliquot equivalent to 2000 units was injected into each of 50 mice followed thirty minutes later by *E. typhosa*.

[2] This lot was obtained through the courtesy of the Antibiotic Study Section of the National Institute of Health and was originally contributed by the Commercial Solvents Corporation for study of penicillin impurities.

As will be noted in Table II this penicillinase inactivated lot, although containing the "factor," showed no protection. Thus, the "factor," alone, appears to exert no protective effect.

In order to determine the effect of the addition of crystalline X to crystalline G, 30% and 50% of the former fraction were added to crystalline G and a total of 2000 units injected into each of 50 mice which were then infected with *E. typhosa* (Table II). It will be noted that 2000 units of crystalline material, of which 30% is crystalline penicillin X, is equivalent to somewhat more than 3000 units of crystalline sodium penicillin G and that the protective effect of 2000 units of the crystalline penicillin mixture, of which 50% is crystalline penicillin X, is equivalent to approximately 4000 units of crystalline sodium penicillin G. When the amount of crystalline penicillin X in the mixture was reduced to 10% there was no discernible increase in the activity. Because of the fact that penicillin X when present in sufficient concentration increases the protective effect of penicillin G, all commercial lots used were tested for the presence of penicillin X by an *in vitro* technique. Except where indicated in the tables none of the lots of penicillin studied contained significant amounts of penicillin X.

TABLE II.—EFFECT OF FACTOR ON PROTECTIVE ACTION OF CRYSTALLINE PENICILLIN G

Penicillin Used	Injected Units	No. Mice	Deaths 24 Hr.	Deaths 48 Hr.	Dead, %
Culture only	None	50	39	45	90
Crystalline G	2000	50	22	27	54
Crystalline G	3000	50	13	21	42
Crystalline G	4000	50	5	11	22
Crystalline G & 30% Crystalline X	2000	50	8	15	30
Crystalline G & 50% Crystalline X	2000	50	7	12	24
Amorphous Lot FP-21 *Assay* Zero u./vial made up to 100,000 units with crystalline penicillin G	2000	50	16	21	42
Amorphous Lot SD-5C *Assay* Zero u./vial made up to 100,000 units with crystalline penicillin G	2000	50	12	15	30
Amorphous Code 19 *Assay* 27,000 u./vial made up to 100,000 units with crystalline penicillin G	2000	50	8	11	22
Amorphous Lot 782H39A[a] completely inactivated with clarase. No penicillin added	None	50	41	43	86

[a] "Factor" shown to be present previously.

Preliminary studies showed that the "factor" was not significantly affected by heating at 100° for twenty-four or forty-eight hours, a process that inactivated the penicillin present. *In vitro* tests with heat inactivated "Code 19" against *E. typhosa* and *S. aureus* at five times the concentration used in the *in vivo* test showed it to be devoid of activity. Using a heat inactivated lot of penicillin known to contain the "factor" (Code 19) a study was made of the effect of the addition to this lot of the various penicillin fractions available. The results are shown in Table III. The addition of the "factor," represented by inactivated "Code 19," to the four peni-

TABLE III.—EFFECT OF ACCESSORY-PROTECTIVE FACTOR ON CRYSTALLINE PENICILLIN FRACTIONS

Penicillin Used	Injected Units	No. Mice	Deaths 24 Hr.	Deaths 48 Hr.	Dead, %
Culture only	None	50	45	47	94
Crystalline G	2000	50	19	28	50
Crystalline G	3000	50	12	17	34
Crystalline G	4000	50	7	9	18
Code #19[a] + Crystalline G	2000	50	9	16	32
Crystalline K	2000	50	32	41	82
Code #19 + Crystalline K	2000	50	18	34	68
Crystalline Dihydro F	2000	50	25	30	60
Code #19 + Crystalline Dihydro F	2000	50	11	14	28
Crystalline X	2000	50	6	8	16
Code #19 + Crystalline X	2000	50	3	3	6

[a] Code #19 inactivated at 100° for 24 hr. before crystalline fractions were added.

TABLE IV.—DISTRIBUTION OF ACCESSORY-PROTECTIVE FACTOR IN AMORPHOUS PENICILLIN

Manufacturers Code	Potency, U./Mg.	Date of Mfr.	A. = P. Factor Present	A. = P. Factor Absent	2000 Units Amorphous Equiv. to:	Enhancement, %
1-P-1	500	3 '44	+	..	3000	50
1-P-1(RPT)	500	3 '44	+	..	3500	75
SD5C	374(s)[a]	9 '44	+	..	3000	50
SD5C(RPT)	374(s)[a]	9 '44	+	..	3000	50
SD6C	462(s)[a]	9 '44	+	..	3000	50
45050902	594	5 '45	+	..	3000	50
45050902(RPT)	594	5 '45	+	..	3000	50
44031901	264	3 '44	+	..	3000	50
44031901(RPT)	264	3 '44	+	..	3000	50
.....	300	...	+	..	3000	50
45051002	561	5 '45	+	..	3000	50
45061802	584	6 '45	..	−	2000	0
46100802(Crystalline)	1524	10 '46	..	−	2000	0
45051002	561	5 '45	+	..	3000	50
99026	821	7 '44	..	−	2000	0
32531	687	9 '44	..	−	2000	0
3646	1074	1 '47	..	−	2000	0
Pooled lots	900	...	+	..	3000	50
G504A015	900	6 '45	..	−	2000	0
G403A051[b]	160	3 '44	+	−	4000	100
62	680	3 '44	+	..	3000	50
32	506	2 '44	..	−	2000	0
SN-101	864(s)[a]	5 '44	+	..	3000	50
77	757(s)[a]	3 '44	+	..	3500	75
2796-C	1250	12 '46	..	−	2000	0
782H39A[b]	320	3 '45	+	..	4000	100
782H39A(RPT)	320	3 '45	+	..	4000	100
9169X341142	344	3 '44	+	..	4000	100
4092-409943	1035	5 '46	..	−	2000	...
EW-762	190	3 '44	+	..	4000	100
EW-762 (RPT)	190	3 '44	+	..	3500	75
AW-679	222	3 '44	..	−	2000	0
287	662(s)[a]	5 '45	+	..	3000	50
FP-21	396(s)[a]	7 '44	+	..	3000	50
248	320	5 '45	+	..	4000	100
1365013	711	9 '46	+	..	3000	50
111	767	8 '44	..	−	2000	0
139C2	940	1 '45	..	−	2000	0
184B1	1160	3 '45	..	−	2000	0
171C1	1300	3 '45	..	−	2000	0
238B1	1460	5 '45	..	−	2000	0
218B1	1120	5 '45	..	−	2000	0
92	525	12 '43	+	..	3000	50

[a] Lots designated "s" had been held at 37° for some time in stability studies. All were returned to original potency by the addition of crystalline penicillin G.
[b] Contains more than 10% penicillin X.
[c] Some enhancement in activity noted.

TABLE V.—DISTRIBUTION OF ACCESSORY-PROTECTIVE FACTOR IN RECENTLY MANUFACTURED LOTS OF PENICILLIN

Manufacturers Code	Potency, U./Mg.	Date of Mfr.	A. = P. Factor Present	A. = P. Factor Absent	2000 Units Amorphous Equiv. to:	Enhancement, %
861P1	956	1 '47	+	..	3000	50
46100802	1524	10 '46	..	—	2000	0
1032	920	12 '46	+	..	3000	50
3646	1074	1 '47	..	—	2000	0
612A001	1171	1 '47	..	—	2000	0
2796C	1250	1 '47	..	—	2000	0
3810-20307	923	1 '47	+	..	3000	50
4092-409943	1035	5 '46	..	—	2000	0
864	1162	4 '46	..	—	2000	0
1299	1248	12 '46	+	..	3000	50
12466	1246	12 '46	+	..	3000	50
15480	610	3 '46	..	—	2000	0

cillin fractions tested markedly increased their protective effect. Two thousand units of crystalline penicillin G or dihydro F when added to the "factor" had a protective effect equivalent to 3000 units of crystalline penicillin G alone. It is of interest that although crystalline penicillin K alone was relatively ineffective in its protective action against *E. typhosa*, the addition of the "factor" present in "Code 19" increased its activity to a point approaching the effectiveness of crystalline penicillin G alone. These data emphasize the difficulties in interpreting the therapeutic efficacy of penicillin fractions in commercial penicillin by *in vitro* or *in vivo* studies with "pure" crystalline penicillin fractions. It would appear that the therapeutic activity of the various fractions in a commercial lot of penicillin would be materially affected by the presence or absence of the "factor." The superior effectiveness of penicillin X is again demonstrated in Table III where it will be noted that 2000 units of crystalline penicillin X is equivalent to 4000 units of crystalline G. In spite of this marked effectiveness of crystalline penicillin X, however, the addition of the "factor" further enhances its activity.

In order to determine the percentage of lots of commercial penicillin that contain the "factor," random samples obtained from the basic manufacturers have been tested. Both recent and relatively old lots of penicillin have been examined and the results are given in Tables IV and V. A commercial lot was not considered to contain the "factor" unless it possessed at a concentration of 2000 units an activity, when measured by the method described, at least 50% in excess of that possessed by 2000 units of crystalline penicillin G. Of the 35 relatively old lots of commercial penicillin tested 21% or 60% were found to contain the "factor." Of the recent lots tested the "factor" was found in 5 (40%) of the 12 lots examined. There appears to be no direct relationship between the potency in u./mg. and the presence or absence of the "factor" since it will be noted that Lot 1299 with a potency of 1248 u./mg. has the "factor" while Lot AW-679 at a potency of 222 u./mg. does not. The process of manufacture may determine the presence or absence of the "factor" in commercial penicillin. This is illustrated by Codes 111, 139C2, 184B1, 171C1, 238B1, and 218B1 produced by the same manufacturer between August, 1944 and May, 1945. None of these lots which represent a fair distribution of the penicillin produced by this manufacturer during this period contain the "factor." However, two recent lots, one of which was produced in 1947, were found to contain the "factor." At present nothing is known of the nature of this accessory protective "factor" nor its mode of action.

SUMMARY

1. A method is presented for determining the presence of a "factor" in penicillin which markedly enhances penicillin activity while being devoid of activity *in vivo* itself.

2. The "factor" is not inactivated by heating at 100° for forty-eight hours nor by penicillinase.

3. The "factor" enhances the effectiveness of each of the fractions G, F, X, K, and dihydro F proportionately to their basic *in vivo* activity.

4. Examination of random samples of relatively old commercial lots of penicillin produced by the 15 basic manufacturers indicate that approximately 60 per cent contain the "factor."

5. Examination of a single lot of recent manufacture (amorphous penicillin) from 11 producers indicates that approximately 40 per cent contain the "factor."

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Determination of Nicotinic Acid and Riboflavin in Multi-Vitamin Tablets*

By LOUIS FEINSTEIN and WILLIAM E. SCOTT

Procedures for determining nicotinic acid (or its amide) and riboflavin in multi-vitamin tablets are described in which certain of the sources of error usually encountered have been largely eliminated.

IN ASSAYING multi-vitamin tablets or capsules for nicotinic acid and riboflavin by colorimetric and fluorometric procedures, respectively, the authors have made certain modifications in the usual procedures which appear to have largely eliminated some of the principal sources of error. In each instance the assay is based on a comparison of the color or fluorescence produced by chemical action in a solution of the material being assayed, with that produced in a similar solution to which has been added a known quantity of pure nicotinic acid or riboflavin. By modifying each procedure in such a manner that the two readings are taken at approximately the same intensity of color or fluorescence, errors due to failure of the reactions to conform strictly to Beer's law and to differences in the sensitivity of the photoelectric instruments used, at different intensities of color and fluorescence, are largely eliminated.

Nicotinic acid has been determined by means of its colorimetric reaction with cyanogen bromide and a primary or secondary amine. The method is based on the principle that pyridine derivatives give specific colors with these reagents (1). The nicotinic acid in many natural products has been determined by this reaction by using aniline as the amine (2–4). This color follows Beer's law when a 420 mμ filter is used in the colorimeter.

Riboflavin can be determined by its characteristic fluorescence in solution which reaches a maximum at a wave length of approximately 450 mμ and at a *p*H of 6 (5, 6). The fluorescence is directly proportional to the riboflavin content. Riboflavin has been determined indirectly by its fluorescence, both before and after reduction with sodium hydrosulfite (7). Reduced riboflavin does not show the typical fluorescence of the unreduced vitamin.

* Received March 27, 1947, from the Grain Branch, Production and Marketing Administration, United States Department of Agriculture, Washington, D. C.

EXPERIMENTAL

Determination of Nicotinic Acid (or Nicotinic Acid Amide as Nicotinic Acid) in Multi-Vitamin Tablets.

The procedure herein described is intended for tablets or capsules believed to contain about 10 mg. of nicotinic acid or its amide, but it may be readily modified for use with tablets or capsules of any other potency.

1. Extraction and Hydrolysis.—Weigh accurately 25 tablets and grind them finely. Place into a 100-ml. volumetric flask (*X*) a portion of the ground tablets equal in weight to 5 tablets and 50 mg. of pure nicotinic acid. (If gelatin capsules are to be assayed, put 5 whole capsules into the flask instead of the ground material.) Into a second flask (*Y*) place a ground sample equal in weight to 10 tablets (or 10 whole capsules). Fill each flask to the mark with 2.0 *N* sodium hydroxide. Shake mechanically for thirty minutes and then centrifuge at 1800 r. p. m. for ten minutes. To 2 ml. of the clear supernatant liquid in a 100-ml. volumetric flask add 15 ml. of 2.0 *N* sodium hydroxide and heat for thirty minutes in a boiling water bath to hydrolyze any nicotinic acid amide to the free acid. Neutralize after cooling to a *p*H of 7 using hydrochloric acid. Fill each flask to the 100-ml. mark with distilled water and centrifuge or filter the solution, discarding the first 20 ml. of filtrate.

2. Color Development.—To 2-ml. portions of each of the above solutions in large diameter test tubes add quickly, with slight shaking at each addition, 5 ml. of buffer solution (see following paragraph), 1 ml. of 4% aniline in 95% alcohol solution, and 6 ml. of 4% cyanogen bromide solution in the order listed. Place the mixture in each instance at once into the colorimeter cells and using a 420 mμ filter read in the photoelectric colorimeter at the point of maximum color development (minimum light transmission) by use of a solution consisting of 2 ml. of distilled water, 5 ml. of the buffer solution, 1 ml. of 4% aniline in 95% alcohol solution and 6 ml. of 4% cyanogen bromide solution to set the colorimeter for zero density. To determine the effect of any color in the solution due to substances other

than nicotinic acid, use 2 ml. of solution from the second flask and 12 ml. of water acidulated with hydrochloric acid to a *p*H of 4. Compare this with 2 ml. of distilled water and 12 ml. of water acidulated with hydrochloric acid to a *p*H of 4 set for zero density. This residual color density equals $2c$ in the formula below.

The buffer solution used above is made by mixing 50 ml. of 0.2 *M* potassium phosphate (primary) with 29.54 ml. of 0.2 *M* sodium hydroxide and diluting to 200 ml. This solution has a *p*H of 7.0.

3. Calculation (Using Klett-Summerson Readings).—Let:

x = color density of solution prepared from that originally in flask *X*

y = color density of solution prepared from that originally in flask *Y*

a = color density (due to added known quantity of pure nicotinic acid)

b = color density (due to unknown quantity of nicotinic acid from tablets or capsules)

c = residual color density from 5 tablets (due to substances other than nicotinic acid)

Then:

$$x = a + b + c$$
$$y = 2b + 2c$$

$$\frac{b}{a} \times \frac{\text{mg. of nicotinic acid added to flask } X}{\text{number of tablets in flask } X} = \text{mg. of nicotinic acid per tablet}$$

$$\frac{b}{a} \times 10 = \text{mg. of nicotinic acid per tablet for the conditions given}$$

NOTE: If, for any reason, color densities cannot be read at once, at the maximum color development, the tubes containing the solutions should be placed in the dark but the color intensities should be read as promptly as possible. Although fading will take place, the rate of fading will be approximately the same in both tubes so that up to one hour the nicotinic acid values obtained will be within 10% of those obtained when the color intensities are read at the time of maximum color development. (See Table I.)

TABLE I.—NICOTINIC ACID AND/OR AMIDE IN TABLETS DETERMINED BY READING COLOR AT VARIOUS TIME INTERVALS AFTER MAXIMUM DEVELOPMENT

(Results Are in Milligrams per Tablet)

Sample	Maximum Color (within 2 Min.)	Time in Min. after Maximum Color Development: 15	30	45	60
8073[a]	10.9	9.1	9.2	9.1	8.8
8072[b]	11.1	10.7	10.3	10.3	10.1

[a] Colored solutions were allowed to stand unprotected from light.

[b] Colored solutions were kept in dark except at time of reading.

Results obtained by the colorimetric method of assay for nicotinic acid are in good agreement with those obtained microbiologically. This is illustrated by the data in Table II. Table III shows that good reproducibility may be obtained with the described colorimetric procedure.

TABLE II.—COMPARISON OF CHEMICAL AND MICROBIOLOGICAL DETERMINATIONS OF NICOTINIC ACID AND/OR AMIDE IN MULTI-VITAMIN TABLETS

Sample	Chemical, Mg. per Tablet	U. S. P. Microbiological,[a] Mg.	Chemical as Percentage of Microbiological
549	10.87	10.7	101.6
15397	10.45	10.2	102.4
15591	11.27	11.2	100.6

[a] Determinations made by another laboratory.

TABLE III.—CHEMICAL DETERMINATIONS OF NICOTINIC ACID AND/OR AMIDE IN TABLETS ANALYZED ON DIFFERENT DAYS—REPRODUCIBILITY OF METHOD

Sample	Mg. per Tablet: Trial I	Trial II
8072	10.98	11.10
10038	11.16	10.90
10186	10.43	10.32
10576	10.71	10.71
13570	11.95	11.25

Determination of Riboflavin in Multi-Vitamin Tablets.

The procedure herein described is intended for tablets or capsules believed to contain about 1.0 mg. of riboflavin, but it may be readily modified for use with tablets or capsules of any other potency.

1. Preparation of Diluted Sample.—Weigh accurately 20 tablets and grind them to a fine powder, being careful to protect the ground material from light, and weigh out a quantity equal in weight to 5 tablets. Gelatin capsules are used without grinding. Place this weighed portion (or 5 capsules) into a 300-ml. Erlenmeyer flask with 100 ml. of 0.1 *N* sulfuric acid and autoclave at 15-lb. pressure for twenty minutes. After cooling, dilute the resulting solution to 500 ml. with 0.1 *N* sulfuric acid in a volumetric flask. To 5 ml. of this diluted solution add 12.5 ml. of 2.5 *N* sodium acetate solution and dilute to 100 ml. with 0.1 *N* sulfuric acid. Protect the solution from light during the preparation.

2. Determination.—To two 50-ml. glass-stoppered cylinders designated as *A* and *B* (place a roll of dark paper around each cylinder to protect the solution from light or use low actinic glassware) add in order as follows:

Cylinder *A*

(*a*) 10 ml. of the solution prepared under (1) above.

(*b*) 10 ml. of acetone-water solution (5 ml. of distilled water diluted to 100 ml. with acetone).

(*c*) 1 ml. of 0.333 *N* acetic acid solution. Mix.
(*d*) 0.5 ml. of 4% potassium permanganate solution (freshly made). Mix and let stand for two minutes.
(*e*) 0.5 ml. of 3% hydrogen peroxide (freshly made from 30% solution). Mix.
(*f*) Dilute to 25 ml. with acetone.

Cylinder *B*

(*a*) 10 ml. of the solution prepared under (1) above.
(*b*) 10 ml. of riboflavin solution in acetone (5 ml. of a solution containing 10 γ of riboflavin per ml. diluted to 100 ml. with acetone).
(*c*) 1 ml. of 0.333 *N* acetic acid solution. Mix.
(*d*) 0.5 ml. of 4% potassium permanganate solution (freshly made). Mix and let stand for two minutes.
(*e*) 0.5 ml. of 3% hydrogen peroxide (freshly made from 30% solution). Mix.
(*f*) Dilute to 25 ml. with acetone.
(*g*) Dilute to 50 ml. with a solution containing (*a*) 87.5 ml. 0.1 *N* sulfuric acid, (*b*) 12.5 ml. of 2.5 *N* sodium acetate solution, (*c*) 5.0 ml. of freshly prepared 4% potassium permanganate solution, (*d*) 5.0 ml. of 3% hydrogen peroxide solution, (*e*) 10 ml. of 0.333 *N* acetic acid solution, (*f*) 5.0 ml. of distilled water, and (*g*) 125 ml. of acetone. This solution need not be made freshly each time it is used.

If the solutions in cylinders *A* and *B* are not clear, centrifuge them for five minutes at 1500 r. p. m. Read solutions *A* and *B* in a photoelectric fluorescence meter with proper filters (Coleman model No. 12 or equivalent). Add approximately 0.01 Gm. of sodium hydrosulfite to solution *A* and shake. Re-read fluorescence as *C*.

3. Calculation (Using Meter Readings of Coleman Instrument).

$$\frac{A - C}{2B - A} = \text{Milligrams of riboflavin per tablet}$$

The data in Table IV are presented to show a comparison of the amount of riboflavin found to that required to be present in multi-vitamin tablets being supplied to the Government on contract. These tablets had to meet the requirement of having 1 mg. of riboflavin per tablet after being stored at 45° for 500 hours.

TABLE IV.—DETERMINATION OF RIBOFLAVIN IN MULTI-VITAMIN TABLETS AFTER STORAGE AT 45° C FOR 500 HOURS

Sample	Mg. per Tablet Found	Required
2517	1.12	1.0
3570	1.04	1.0
6430	1.03	1.0
6431	1.03	1.0
16491	1.03[a]	1.0
16619	1.07[a]	1.0

[a] 1.11 and 1.11, respectively, before storage.

SUMMARY

A colorimetric procedure for determining nicotinic acid (or its amide) and a fluorometric procedure for determining riboflavin in multi-vitamin tablets are described in which certain of the sources of error usually encountered in determinations of these types have been largely eliminated. In both procedures intensity of color or fluorescence of solutions of the material under assay is compared with that of similar solutions to which have been added a known quantity of the pure reacting agent. The procedures are so devised that the color or fluorescence of both solutions is read at approximately the same level of intensity. In this way errors due to failure of the reactions to conform strictly to Beer's law and to inaccuracies in the photoelectric instruments used to make the color or fluorescence readings, are largely eliminated.

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The Microbiological Determination of Proline, Cystine, and Isoleucine*

By GERSHON L. HELLER† and ERNST R. KIRCH

The basal medium proposed by Dunn, *et al.*, for the microbiological determination of amino acids using *Leuconostoc mesenteroides P-60* has been modified so as to increase the acid production. The arginine content of the medium has been increased five-fold, the alanine content reduced by one-half; norleucine, norvaline, as well as hydroxyproline, have been omitted. The proposed medium has applied to the determination of proline, cystine, and isoleucine in various hydrolyzed proteins. Recovery experiments gave results falling within the limits of error for the microbiological assays in general.

SNELL, STRONG AND PETERSON (1), in 1937, working on growth factors for various organisms reported the use of *Leuconostoc mesenteroides P-60* as a lactic acid-producing organism. In 1944, Dunn, *et al.* (2), investigating the amino acid requirements of this organism presented a medium for the determination of lysine (3) utilizing *Leuconostoc mesenteroides P-60* as the test organism and suggesting its use for the determination of twelve other amino acids. We have developed a medium for the determination of proline, cystine, and isoleucine based on this original medium reported by Dunn, *et al.* (2).

EXPERIMENTAL

The organism[1] was carried on Difco's Yeast Dextrose Agar stabs; subcultures were made each week.

Standard solutions of *l*(−) proline and *l*(−) cystine were prepared containing 20 γ per ml. and of *dl*-isoleucine containing 100 γ/ml.

The basal media employed in this investigation are shown in Table I. The particular amino acid assayed, *l*(−) proline, *l*(−) cystine or *dl*-isoleucine is omitted from the medium. The basal medium is prepared double the strength of that shown in Table I. It is adjusted to a *p*H of 6.8 and is sterilized by autoclaving for 10 minutes at 15-lb. pressure. Two and one-half milliliters of basal medium are used for each tube diluted to a final volume of 5 ml. A stock solution containing all of the amino acids in the basal medium excepting *l*(−) proline, *l*(−) cystine and *dl*-isoleucine is prepared and used for the assay of any one of the three amino acids by adding the other two in the levels needed.

The inoculum is grown in tubes containing 2.5 ml. of double strength basal medium and 2.5 ml. of a standard solution of the amino acid being assayed at a concentration equal to the highest level used in the determination of the amino acid. The tubes are incubated for twenty-four hours at 37°. One drop of a saline suspension of the organism is used to inoculate the assay tubes. Standard curves are prepared with range of 0.0 to 50 γ per tube for *l*(−) proline and *l*(−) cystine and 0.0 to 200 γ per tube for *dl*-isoleucine. The assay tubes are incubated for seventy-two hours at 37°. The lactic acid produced is titrated with 0.05 *N* sodium hydroxide using bromthymol blue as the indicator. Figure 1 represents typical reference curves obtained using Dunn's (2) and our media.

The proteins used in this investigation were all hydrolyzed by refluxing for eight hours with 80 ml. of 8 *N* hydrochloric acid (4). The solution was neutralized with sodium hydroxide to a *p*H of 6.8, diluted to the proper volume and kept under toluene in the refrigerator. Recovery experiments were conducted for *l*(−) proline on gelatin, blood albumin, egg albumin, casein and gliadin (Table II); for *l*(−) cystine on egg albumin, blood albumin and gliadin (Table III); and for *l*-isoleucine on casein, egg albumin, blood albumin and gliadin (Table IV). The amino acid content of the proteins was determined on dilutions of the hydrolyzates at different levels. Recoveries of added amounts of these amino acids were made at the same levels. Table V shows the percentages of *l* (−) proline, *l*(−) cystine and *l*-isoleucine in the above-mentioned proteins that were found using the modified basal medium herewith presented as compared with previously published results.

DISCUSSION

The need for further study of the amino acid levels in basal media has been shown in the literature as well as in actual laboratory procedure. Barton-Wright, *et al.* (5) have

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† This paper is based on a thesis submitted by Gershon L. Heller to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Master of Science.

[1] *Leuconostoc mesenteroides* 8042 American Type Culture, E. E. Snell, University of Texas, Strain P-60.

TABLE I.—COMPOSITION OF BASAL MEDIA

Constituent	Dunn, *et al.* (2) Medium D, Mg./L.	Dunn, *et al.* (2) Medium D (Tripled), Mg./L.	Barton-Wright *et al.* (5) Medium, Mg./L.	Modified Medium Mg./L.
dl-Alanine	2000	6000	1000	1000
l (+) Arginine·HCl	80	240	250	400
l-Asparagine	400	1200	800	400
l (−) Cystine	120	360	100	120
l (+) Glutamic Acid	150	450	500	150
Glycine	100	300	100	100
l (−) Histidine·HCl·H_2O	20	60	100	20
l (−) Hydroxyproline	100	300	...	...
dl-Isoleucine	150	450	200	150
l (−) Leucine	75	225	100	75
l (+) Lysine·HCl	160	480	250	160
dl-Methionine	40	120	100	40
dl-Norleucine	100	300	100	...
dl-Norvaline	100	300	100	...
dl-Phenylalanine	60	180	100	60
l (−) Proline	25	75	100	25
dl-Serine	80	240	100	80
dl-Threonine	450	1350	500	450
l (−) Tryptophane	10	30	100	10
l (−) Tyrosine	30	90	100	30
dl-Valine	150	450	200	150
Glucose	20 (Gm.)	20 (Gm.)	20 (Gm.)	20 (Gm.)
Adenine Sulfate	12	12	12	12
Guanine Hydrochloride	12	12	12	12
Uracil	12	12	12	12
Sodium Acetate	12 (Gm.)	12 (Gm.)	12 (Gm.)	12 (Gm.)
NH_4Cl	6 (Gm.)	6 (Gm.)	6 (Gm.)	6 (Gm.)
KH_2PO_4	500	500	500	500
K_2HPO_4	500	500	500	500
$MgSO_4 \cdot 7H_2O$	200	200	200	200
NaCl	10	10	10	10
$FeSO_4 \cdot 7H_2O$	10	10	10	10
$MnSO_4 \cdot 4H_2O$	10	10	10	10
Thiamin Hydrochloride	1	1	1	1
Pyridoxine	1.6	1.6	1.6	1.6
dl-Calcium Pantothenate	2	2	2	2
Riboflavin	2	2	2	2
Nicotinic Acid	2	2	2	2
Biotin	5 (γ)	5 (γ)	5 (γ)	5 (γ)
Folic Acid	2 (γ)	2 (γ)	2 (γ)	2 (γ)
p-Aminobenzoic Acid	0.1 (γ)	0.1 (γ)	0.1 (γ)	0.1 (γ)
Adjust to *p*H 6.8				
Add distilled water to	1000 ml.	1000 ml.	1000 ml.	1000 ml.

TABLE II.—RECOVERIES OF *l* (−) PROLINE

Material	Proline Content, γ/Tube	Proline Added, γ/Tube	Total, γ/Tube	Proline Recovered, γ/Tube	Recovery, %
Gelatin	16.93	20	36.93	39.67	107
	26.60	10	36.60	35.78	98
	33.96	10	43.96	44.29	101
Blood Albumin	7.59	20	27.59	30.00	108
	18.25	20	38.25	40.33	105
	26.27	10	36.27	37.26	103
	36.11	10	46.11	47.59	103
Egg Albumin	5.28	20	25.28	24.79	98
	11.98	20	31.98	30.33	95
	18.42	10	28.42	28.25	99
	25.28	10	35.28	33.80	96
Casein	9.41	20	29.41	28.58	97
	18.42	20	38.42	38.41	100
	27.59	10	37.59	34.29	91
	38.58	10	48.58	46.93	97
Gliadin	13.30	20	33.30	34.62	104
	24.13	20	44.13	46.57	105
	37.43	10	47.43	49.57	104
	49.24	10	59.24	58.75	99

reported the inadequacy of Dunn's (2) medium (Table I) for this purpose and the low acid production has also been experienced by the authors of this report. Dunn, in further reports (6, 7) has increased the amino acid levels in his basal medium 2.5 times and has decreased the concentration of the sodium hydroxide used in titrations from 0.1 *N* to 0.05 *N* also recognizing the inadequacy of his original medium (Table I). He recommended, in a still later report (8), the tripling of the amino acid levels. Higher acid production and extended linear segments of the standard curve were the results of this modification. However, since a basal medium should contain the minimal quantity of each amino acid required to give the maximal growth (2) it was deemed inadvisable to make a complete threefold increase in all of the amino acids. This investigation was therefore undertaken to determine which of the individual amino acids in increased levels caused the higher production of acid and extended the linear segments of the standard curve. After a study of results obtained by a series of modifications in Dunn's medium (Table I), the modified basal medium presented in this report (Table I) was developed. The essential differences in the two media are that hydroxyproline, norleucine and norvaline are omitted, the level of alanine is half of that originally employed and the level of *l* (+) arginine mono-

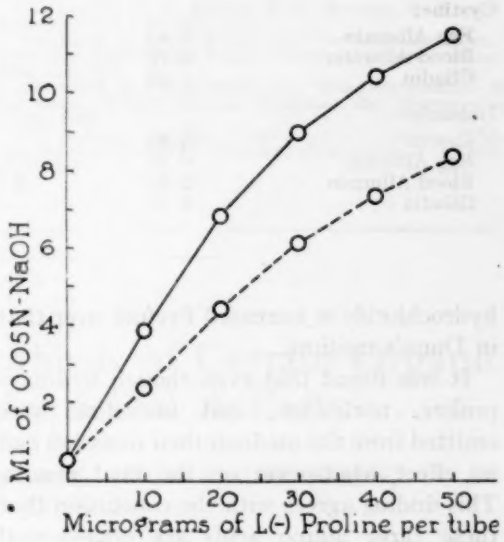

Fig. 1.—Typical curves obtained for *l*(−) proline using *Leuconostoc mesenteroides P-60*. Broken line represents the curve obtained with Dunn's medium, solid line the curve obtained using the author's modification.

TABLE III.—RECOVERIES OF *l* (−) CYSTINE

Material	Cystine Content, γ/Tube	Cystine Added, γ/Tube	Total, γ/Tube	Cystine Recovered, γ/Tube	Recovery, %
Egg Albumin	10.99	20	30.99	32.15	104
	27.43	10	37.43	33.96	91
	29.24	10	39.24	37.10	95
Blood Albumin	5.94	20	25.94	23.30	90
	17.92	10	27.92	23.30	84
	19.41	10	29.41	25.28	86
Gliadin	7.59	20	27.59	28.58	103
	14.13	20	34.13	30.99	91
	16.60	10	26.60	23.63	90

TABLE IV.—RECOVERIES OF *l*-ISOLEUCINE

Material	Isoleucine Content, γ/Tube	Isoleucine Added, γ/Tube	Total, γ/Tube	Isoleucine Recovered, γ/Tube	Recovery, %
Casein	14.33	10	24.33	22.66	93
	20.00	10	30.00	31.00	103
	26.65	5	31.65	32.33	102
Egg Albumin	13.33	10	23.33	22.33	96
	18.32	10	28.32	29.33	104
	24.66	5	29.66	30.00	101
Blood Albumin	2.00	50	52.00	56.32	108
	4.33	50	54.33	56.99	105
	5.32	25	30.32	32.66	108
Gliadin	14.66	50	64.66	67.65	105
	20.67	50	70.67	70.34	99
	28.32	25	53.32	53.66	100

TABLE V.—PERCENTAGES OF *l* (−) PROLINE, *l* (−) CYSTINE AND *l*-ISOLEUCINE IN VARIOUS PROTEINS (Compared With Literature Values)

	Authors' Value	Literature Value	Reference No.	Method Used
Proline:				
Gelatin	13.79	15.3 ± 0.4	10	Bergmann
Blood Albumin	1.72	1.0	20	
Egg Albumin	2.37	4.3	11	Dakin
Casein	9.36	7–9	12	Dakin–Van Slyke
Gliadin	12.54	12	13	Fischer
Cystine:				
Egg Albumin	1.83	1.8	14	Schulz
Blood Albumin	3.73	3.6	15	Hopkins–Graff
Gliadin	1.35	1.6	16	Folin
Isoleucine:				
Casein	6.84	6.5	17	Lyman
Egg Albumin	6.31	5	19	
Blood Albumin	2.52	2.7 ± 0.2	18	Fromageot–Block
Gliadin	4.73			

hydrochloride is increased fivefold over that in Dunn's medium.

It was found that even though hydroxyproline, norleucine, and norvaline were omitted from the med.um their omission had no effect whatsoever on the final results. This finding agrees with the conclusion that these three amino acids are nonessential growth factors of *Leuconostoc mesenteroides P-60* (2). However, if significant amounts of these acids are present as impurities in the other amino acids used in the medium this conclusion would not be valid (2).

In concurrence with Barton-Wright, *et al.* (5) it was found that the level of alanine could be lowered to one-half of its original level in Dunn's medium (Table I). This finding is in accord with the views of Snell, *et al.* (9). They found that alanine may serve as a direct precursor for pyridoxine. Since this medium contains pyridoxine, the alanine concentration may well be reduced to a lower level than that recommended by Dunn.

The fivefold increase of the level of *l* (+) arginine monohydrochloride gave a standard curve of steeper slope and an extended linear segment and with a definite increase of acid production (from 8.5 ml. with Dunn's medium to 12 ml. with the modified medium). All the blanks in the determinations were under 1 ml. Also the standard curves obtained in the determination of each amino acid (proline, cystine, and isoleucine) were practically superimposable with the other standard curves obtained in the different determinations of that individual amino acid. Percentages of the amino acids found in the various proteins analyzed in this investigation compared favorably with previously published results (Table V). Recovery runs of the amino acids on these proteins were all quantitative within the experimental error of microbiological methods (Tables II–IV).

Barton-Wright, *et al.* (5) have also presented a modification of the medium reported by Dunn, *et al.* (2). Their modified medium is also shown in Table I. Although the results obtained with it are comparable to ours, the larger increases in the levels of most of the amino acids seem to be unwarranted.

The modified basal medium presented in this report can undoubtedly be extended in its use for the determination of other of the amino acids essential for *Leuconostoc mesenteroides P-60*, merely by the omission of the particular amino acid from this basal medium.

SUMMARY

A modification of the basal medium of Dunn, *et al.* (2) has been developed which increases the acid production and gives reproducible and closely linear standard curves using *Leuconostoc mesenteroides P-60* for the determination of proline, cystine and isoleucine.

The modified basal medium has been applied to the determination of proline, cystine,

and isoleucine in various proteins and results of recovery experiments of added amounts of these amino acids to these proteins are presented.

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The Study, Testing, and Synthesis of Certain Organic Compounds for Rodenticidal Activity*

By JOHN W. NELSON,† L. D. EDWARDS, JOHN E. CHRISTIAN, and GLENN L. JENKINS

Thirty-two known compounds were tested as rodenticides. Of all the known compounds tested DDT was the most toxic to rats. A series of eleven compounds similar in structure to DDT was prepared and tested for rodenticidal activity. Four of the eleven compounds synthesized are new and six were prepared by a new method. As a rodenticide only trichloromethyl-(triazyl-amino(2)-)-methanol appears to merit further investigation.

IN SPITE OF all that has been done to combat the rat, this pest is still mankind's greatest enemy (1). Losses from depredations of rats amount to more than those from all other injurious mammals combined. Not only are rats a cause of damage and destruction of food and property, but they also constitute a constant menace to the health of man. It is stated (2) that the rat has been responsible for more untimely deaths among human beings than all the wars of history.

The enormous demand for more efficient means of rat destruction provides a ready field for the exploitation of an endless variety of control methods. Of the methods for the destruction of rats, poisoning is the most efficient and successful. The principal requirements for an ideal rat poison are safety, effectiveness, and economy. The rat poisons in most general use are arsenic, barium carbonate, phosphorus, strychnine, thallium, and red squill.[1] All, with the exception of red squill, have toxic properties that seriously menace the safety not only of wild and domestic animals, but also of human beings. Red squill more nearly approaches the ideal rat poison in that it is relatively safe, and is effective when properly prepared and distributed. However, red squill is a crude plant product and is subject to all the inherent disadvantages of such a product. And even though it is probably the best and most widely used raticide, a survey of the literature gives ample proof that it is far from the ideal rat poison. Therefore, an investigation of rodenticides seemed logical both from an economic and from a public health standpoint.

* Received March 10, 1947, from the Research Laboratories, Purdue University, School of Pharmacy, Lafayette, Ind.
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[1] This work was performed prior to the release of "ANTU" and "1080."

An extensive list was made of toxic drugs with possible rodenticidal properties, but very little useful information was revealed from this effort. However, two observations were made from this list which seemed pertinent: first, the rat is apparently more sensitive to poisons of the nervous systems; and, secondly, it was noticed that those substances generally used as insecticides seemed to have a peculiarly high toxicity to rats.

Peters (3) makes the statement that hydrofuramide has been proposed as a rat poison, and is claimed to be nontoxic to domestic animals. No other reference to such a use of furfuramide was found.

were fed in bait. Purina Dog Chow Meal, the rats' usual diet, was used as the bait in all tests. Healthy, young albino rats of mixed sex, usually in groups of 6 to 15 for exploratory tests, were weighed, placed in individual cages, and starved for twenty-four hours preceding the feeding.

Since rats will consume at a single feeding a quantity of food equal to about 1% of their body weight, the desired dosage of the poison was contained in an amount of bait determined on this weight basis.

Twenty-four hours after the feeding any remaining bait was removed and weighed to determine the consumption per Kg. of rat, and the rats were returned to their normal diet. After the feeding the animals were kept under observation for 5 days for all symptoms of poisoning.

The results are tabulated in Table I.

TABLE I.—TESTING OF KNOWN COMPOUNDS FOR RODENTICIDAL ACTIVITY

Drug	Calculated Dose Range, Mg./Kg.	Lowest Fatal Dose, Mg./Kg.
Sodium Thiocyanate	715–3180	...
Potassium Thiocyanate	990–4000	...
Methyl Thiocyanate	770–1415	...
Ethyl Thiocyanate	1088–1300	...
n-Butyl Thiocyanate	Rats refused to eat	...
Phenyl Isothiocyanate	Rats refused to eat	...
Dimethyl Phthallate	1169–1760	...
Benzyl Isothiourea	1500–3975	3000
DDT (dry)	510–2550	2400
DDT (in olive oil)	170– 350	200–300
Sodium-5-nitroquinoline-8-sulfonate	930–3150	...
5-Nitroquinoline-8-sulfonamide	1875–3675	2200
5-Nitro-8-chloroquinoline	1800–2850	1750
2,4-Dinitro-chlorobenzene	322– 471	...
p-Nitrochlorobenzene	478– 672	...
2,4,6-Trichlorophenol	973–2790	2000
2-Chloro-5-hydroxy-toluene	1515–1878	...
p-Nitrobenzyl Bromide	388– 560	...
Hexachlorobenzene	1723–3430	...
Pentachlorobenzene	1065–1300	...
Polychlornaphthalene	1050–2280	...
Dichlornaphthalene	935–2220	...
Pentachlorphenylbenzoate	630–2270	2000
5,6-Dichlornicotinic Acid	750–2040	...
Chlorohydroquinone	755–1930	...
Octachloro-dipheno-quinone	840–1555	...
Decachloro-meta-terphenyl-diquinone	940–2170	...
4,4′-Dinitro diphenyl disulfide	Rats refused to eat	...
o-Dichloro dibenzyl disulfide	1538–2180	...
2,2′-4,4′-Tetrachloro dibenzyl disulfide	2290–2880	...
4,4′-Dichloro dibenzyl disulfide	750–2570	...
3,3′-4,4′-Tetrachloro dibenzyl disulfide	1700–2300	...
Furfuramide (250 mg./Gm. of bait)	345–1900	345
Furfuramide (50 mg./Gm. of bait)	339– 589	...
Furfuramide (100 mg./Gm. of bait)	180– 620	180
Furfuramide (150 mg./Gm. of bait)	470–1190	530
Furfuramide (in olive oil) (stomach tube)	80– 360	300

PART I

Thirty-two known compounds of various types were tested toxicologically in an endeavor to find some chemical structures which possessed the desired properties for the syntheses of rodenticides.

Since a rat poison must necessarily be introduced into the animal by mouth, the selected compounds

PART II

The proposed plan for this part of the study was to synthesize a series of compounds similar in structure to 2,2-di(*p*-chlorophenyl)-1,1,1-trichlorethane (DDT). This type of structure was selected for synthesis because it seemed to offer the most promise for a number of reasons. DDT itself was the most toxic

to rats of all the compounds tested. According to the results of Smith and Stohlman (4), it has a certain selectivity in its toxicity to rats.

Any condensation with chloral would necessarily contain chlorine which seems to be a desirable constituent of a rat poison, and from our results it seemed that the chlorine of the chloral was necessary for the characteristic nervous action of DDT. The chief symptomatic action of DDT is definitely on the nervous system and rats are especially sensitive to such poisons. The toxicity of DDT in oil compares very favorably with the toxicity of the best of the generally used rat poisons.

EXPERIMENTAL

1. Preparation of 2,2-di-(2-Chloroanilino)-1,1,1-trichlorethane.—Sumerford and Dalton (5) reported a procedure in which this product was prepared by mechanically shaking over a period of seventy-two hours a mixture of *o*-chloroaniline, chloral hydrate, glacial acetic acid, and sodium acetate. This procedure was followed and the product recrystallized from an ethyl alcohol-water mixture. A 94% yield was obtained; m. p. 104°.

2. Preparation of 2,2-di(Anilino)-1,1,1-trichlorethane.—Using the procedure of Sumerford and Dalton (5), a mixture of aniline, chloral hydrate, glacial acetic acid, and sodium acetate was shaken for seventy-two hours. The white crystalline product obtained was recrystallized from an alcohol-water mixture; yield 88%, m. p. 107°. This compound was prepared by other methods by Wallach (6) and Eibner (7).

3. Preparation of 2,2-di-(3-Chloroanilino)-1,1,1-trichlorethane.—A mixture of *m*-chloroaniline, chloral hydrate, glacial acetic acid, and sodium acetate was shaken for seventy-two hours as previously described. A thick, amorphous, pasty mass was obtained which was purified by crystallization from an alcohol-water mixture; yield 83%, m. p. 89°. Eibner (7) prepared this product by another method.

4. Preparation of 2,2-di-(4-Chloroanilino)-1,1,1-trichlorethane.—Using the same procedure, a mixture of *p*-chloroaniline, chloral hydrate, glacial acetic acid, and sodium acetate was mechanically shaken for twenty-four hours after which time a white crystalline solid was obtained. The product was recrystallized from an alcohol-water mixture; yield 89%, m. p. 143°. This compound was prepared by another method by Eibner (7).

5. Preparation of 2,2-di-(*p*-Hydroxyphenyl)-1,1,-1-trichlorethane.—It was thought that the condensation of chloral with phenol would occur through the hydroxyl group forming an ether linkage as in a diacetal. However, the condensation occurred on the benzene ring instead.

Fifty grams of phenol was placed in a round-bottomed flask along with 50 cc. of benzene and the mixture refluxed until solution occurred. About 10 Gm. of phosphorus pentoxide was then added. A benzene solution of chloral was prepared by adding slowly with mixing phosphorus pentoxide to a benzene solution of 40 Gm. of chloral hydrate. The chloral solution was added to the hot phenol solution slowly through the condenser and the mixture refluxed for four hours, during which it turned a brilliant red color. The benzene solution was decanted and the benzene removed by a stream of air, yielding a thick red liquid. This was allowed to stand for twenty-four hours, during which time crystallization occurred. The fine white crystals were washed with water, recrystallized from benzene, and dried in an oven; yield 22%, m. p. 198°. This product was reported by Yager (8) by another method.

6. Preparation of Trichloromethyl-(pyridyl-amino(2)-)-methanol.—Using the same procedure as in the previous experiment, 2-aminopyridine was refluxed with chloral for two hours. Upon removal of the benzene by a stream of air a white crystalline product was obtained. This was recrystallized from alcohol-water; yield 28%, m. p. 110° from alcohol-water, 106° to 108° from benzene. This product was prepared by Feist (9) using another method.

7. Preparation of Trichloromethyl-di-(pyridyl-amino (2)-)methane.—Vacuum sublimation of the alcohol derivative, trichloromethyl-(pyridyl-amino-(2)-)methanol, yielded the condensation product; m. p. 171°. Feist (9) reported this compound by another method.

8. Preparation of Trichloromethyl-(4-methyl-pyrimidylamino(2)-)methanol.—Using the same procedure as in 5, chloral was refluxed with 2-amino-4-methyl pyrimidine for two hours. The white crystalline product obtained on removal of the benzene was recrystallized from benzene; yield 94%, m. p. 175° to 176° with decomposition (total immersion thermometer; sealed tube). The product showed sublimation properties.

Anal.—Calcd. for $C_7H_8N_3Cl_3O$: N, 16.47%; Cl, 41.17%. Found: N, 16.46%; Cl, 41.15%.

9. Preparation of Trichloromethyl-(pyrimidyl-amino (2)-)methanol.—Chloral was refluxed with 2-amino-pyrimidine following the same procedure as in 5. The white crystals which formed on the removal of the benzene were recrystallized from benzene; yield 92%, m. p. 167° to 169° (total immersion thermometer; sealed tube). Sublimation properties were shown.

Anal.—Calcd. for $C_6H_6N_3Cl_3O$: N, 17.33%; Cl, 43.90%. Found: N, 17.50%; Cl, 43.90%.

10. Preparation of Trichloromethyl-(4,6-dimethylpyrimidyl-amino(2)-)methanol.—Using the same procedure as in 5, chloral was refluxed with 2-amino-4,6-dimethyl pyrimidine. The white crystals obtained were recrystallized from benzene; yield 91%, m. p. 138° to 141° (total immersion thermometer; sealed tube). Sublimation properties were evident.

Anal.—Calcd. for $C_8H_{10}N_3Cl_3O$: N, 15.61%; Cl, 39.03%. Found: N, 15.71%; Cl, 39.41%.

11. Preparation of Trichloromethyl-(thiazyl-amino(2)-) methanol.—Again using the same procedure as in 5, chloral was refluxed with 2-amino-

thiazole for two hours. On removal of the benzene by means of a stream of air, light-tan colored crystals were obtained and purified by recrystallization from benzene; yield 86%, m. p. 145° to 147° with decomposition (total immersion thermometer; sealed tube). The product showed sublimation properties.

Anal.—Calcd. for $C_5H_3N_2Cl_3OS$: N, 11.38%; S, 13.01%; Cl, 43.03%. Found: N, 11.70%; S, 13.13%; Cl, 43.04%.

Since the compounds obtained in 6, 7, 8, 9, 10, and 11 all showed a tendency to sublime, drying was carried out in a vacuum piston at 70°. It seemed logical to expect that sublimation of the alcohol derivative or addition product obtained in 8, 9, 10, and 11 would give a condensation product analogous to that obtained in 7. Vacuum sublimation was carried out, but the expected products were not obtained.

TABLE II.—TESTING OF THE COMPOUNDS SYNTHESIZED

Compound[a]	No. Rats	Calculated Dose Range, Mg./Kg.	No. Killed	No. Survived	Lowest Lethal Dose, Mg./Kg.
1	18	1850–2560	0	18	...
2	16	1870–3550	0	16	...
3	14	612–3150	0	14	...
4	14	1810–2420	0	14	...
5	16	680–2280	3	13	2000
6	16	700–1720	3	13	1700
7	16	1900–2500	0	16	...
8	32	1770–2500	0	32	...
9	32	1740–2500	0	32	...
10	32	1740–2260	0	32	...
11	56	430–1240	41	15	500

[a] Numbers correspond with numbers in Part II.

PART III

The eleven compounds prepared were tested by feeding in Dog Chow Meal as before described. Because of the possibility of oil increasing the absorption and thereby the toxicity, all were fed also in olive oil. However, in this series oil did not change the toxicity appreciably. The results are shown in Table II.

SUMMARY

1. Thirty-two different compounds were tested toxicologically to determine whether they possessed the properties desired for the synthesis of rodenticides.

2. 2,2 - Bis - (*p* - chlorophenyl) - 1,1,1-trichlorethane (DDT) when fed in olive oil was the most toxic of these compounds to rats.

3. Furfuramide when fed in adequate concentrations was found to be sufficiently toxic to warrant further investigation as a rat poison.

4. Eleven organic compounds of the DDT type were prepared and tested for their rodenticidal activity.

5. Six compounds are reported by a new method.

6. Four compounds are reported for the first time.

7. Of the compounds prepared, only trichloromethyl-(thiazyl-amino(2)-)-methanol appears to merit further investigation as a rodenticide.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

3,5-Dibromosalicylaldehyde
2-Methyl-3-hydroxy-4-pyrone
Coniferin
3,4,5-Trihydroxyphenanthrene
Uropterine
Xanthopterine
Erythritol
Pyrithiamin
Lumiflavin
Galactoflavin
Di-α-pyridylamine
Perhydrocyclopentanophenanthrene
Diiodotyrosine-*p*-nitrobenzyl ether
Diiodotyrosine-*p*-nitrophenyl ether
Diiodotyrosine benzyl ether
Ferrous glycinate
Ferrous aspartate
Ethionine
Laudanosine
Pyridazone
Pyridazone-3-carboxylic acid
Canaline

Germicidal Action of Some Benzyl Quaternary Ammonium Compounds Having Substituents in the Aromatic Nucleus*

By C. A. LAWRENCE, C. E. KWARTLER, V. L. WILSON and E. W. KIVELA

The bactericidal and bacteriostatic properties of a homologous series of benzyl quaternary ammonium compounds having substituents in the aromatic nucleus are described. The influence of the number of carbon atoms in the alkyl groups and the introduction of one or two chlorine atoms in the aromatic nucleus on germicidal activity is described. The effect of carbon chain lengths in the alkyl groups of certain benzyl quaternary ammonium compounds upon the surface tension of solutions is also reported. No correlation between surface tension values and antibacterial activity was found.

JACOBS, Heidelberger, and their co-workers reported as early in 1916 (1, 2) on the bactericidal properties of a number of quaternary ammonium salts of hexamethylenetetramine. However, almost twenty years elapsed before any further significant contributions to this field were published. It remained for Domagk (3) to focus attention on surface-active quaternary ammonium compounds as germicides and antiseptics. This group of compounds was of particular interest in that, unlike previously known disinfectants, they exhibited not only germicidal action but detergent wetting properties as well.

The "wetting" action, degree of detergency, or effect on lowering surface tension are not the only factors determining the superiority of one germicide over another. Gershenfeld and Milanick (4) and others (5, 6) have shown that, while many of the more effective cationic detergents have low surface tension, some of the less effective ones are equally surface active. Furthermore, in many cases the anionic compounds of feeble germicidal action have an effect on surface tension comparable to the most active cationic detergent.

Baker, Harrison, and Miller (5) concluded their studies on the effects of a large series of anionic and cationic detergents on the metabolism of bacteria with several generalizations: (*a*) Cationic detergents are equally effective in high dilutions in inhibiting the growth of both Gram-positive and Gram-negative bacteria; (*b*) anionic detergents with but few exceptions selectively inhibit the metabolism of Gram-positive bacteria only; and (*c*) the cationic detergents are more active in the alkaline range of *p*H while the anionic are most effective in the acid range.

The relationship of chemical structure to germicidal activity of various quaternary ammonium salts has been described by various workers (5–9). In general, the chain length of the lipophilic group of cationic as well as anionic detergents has modified the degree of inhibition of the compound. The C_6, C_8, and C_{10} alkyl derivatives of quaternary ammonium compounds have but a feeble action against microorganisms. Compounds with carbon chain lengths of C_{12}, C_{14}, and C_{16} have been most effective, while upon increasing the number of atoms to C_{18}, a marked reduction in activity occurs.

The preparation of the large number of quaternary ammonium compounds that have been described during the past several years was chiefly concerned with the variation of the substituents attached to the nitrogen atom. These new derivatives included the following: (*a*) compounds containing an aromatic ring in the long chain of the type *p*-tertiaryoctylphenoxyethoxyethyldimethylbenzyl-ammonium chloride monohydrate (10); (*b*) compounds in which oxygen atoms are introduced in the long chain as amide linkages of the type lauric acid ester of colaminoformylmethylpyridinium chloride (8); (*c*) compounds containing unsaturated links in the long-

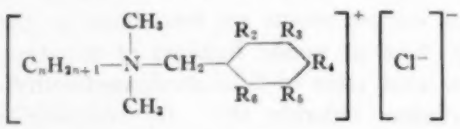
* Received May 26, 1947, from the Winthrop Chemical Company, Inc., Rensselaer, N. Y.

chain alkyl groups of the type 9-octadecenyldimethylbenzyl-ammonium chloride (11); (*d*) those compounds in which two long chain alkyl radicals (C_{12} to C_{16}) are introduced on the nitrogen atom to give a series of dialkylmethylbenzyl-ammonium chlorides (7); and (*e*) cyclic amines, pyridine, picoline, lutidine, etc., of the type cetyl-pyridinium chloride (9).

With the exception of the Jacobs papers (1916), the germicidal activity of quaternary ammonium compounds in which radicals are introduced into the aromatic nucleus has received but little attention. Kolloff, Wyss, Himelick, and Mantele (7) described the germicidal action of several alkyl-pyridinium and alkyl-picolinium quaternary ammonium salts which have a methyl radical in the aromatic nucleus. The presence of the methyl group in the position mentioned did not significantly increase the activity of the compounds.

In studies on a series of quaternary ammonium salts, Rawlins, Sweet, and Joslyn (6) noted that substitution of halogens in the aryl group did not increase the germicidal activity of *p*-tertiaryoctylphenoxyethoxyethyldimethylbenzyl-ammonium chloride and may actually decrease it. Leuchs (12) also described several compounds which contained halogens in the aromatic portion of the molecule. However, no reference was made to the bacteriology of the derivatives. DuBois and Dibblee (13, 14) more recently described the antibacterial effects of an alkyldimethylbenzyl-ammonium chloride which contains chlorine substituents in the 3,4-position on the benzene nucleus. The homologous series of long-chain alkyl derivatives of the latter compound were included in our studies.

The group of alkyldimethylbenzyl-ammonium chlorides prepared in our laboratories in which substitutions were made on the aromatic or benzyl portion of the molecule have the following general formula:



n represents 6, 8, 10, 12, 14, 16, or 18; R_2, R_3, etc., indicate positions in which substitutions may be made in the benzene nucleus. The compounds include 2-chlorbenzyl-, 4-chlorbenzyl-, 2,4-dichlorbenzyl-, 3,4-dichlorbenzyl-, isomeric dichlorbenzyl-isomeric trichlorbenzyl-, and 4-nitrobenzyl-derivatives of alkyldimethyl-ammonium chloride with alkyl radicals ranging from C_8 to C_{18}. These radicals are obtained from the fatty acids of coconut oil. The alkyldimethyl ammonium chlorides in which the benzyl group is unsubstituted were used as a basis for comparing the relative germicidal action of the various derivatives.

EXPERIMENTAL

The quaternary ammonium compounds were prepared by conventional methods of synthesis. The series of alkyldimethyl amines, ranging from C_8 to C_{18}, was prepared by the action of the corresponding alkyl halide on dimethylamine. Whenever the alkyl chlorides were available, an autoclave reaction with dimethylamine was used to secure the desired tertiary amines; in those instances when the alkyl bromides were available, or readily prepared, it was found possible to secure the tertiary amines by working at atmospheric pressure and by use of suitable solvents. In some instances the tertiary amines were made by the action of formaldehyde and formic acid on the primary alkylamine.

For the preparation of the quaternary ammonium compounds, the action of benzyl chloride or substituted benzyl chlorides[1] on the tertiary amines was carried out under a variety of conditions. For some of the quaternary compounds, it was possible to run the reaction at elevated temperature and without the use of a solvent. In other instances, it was found more suitable to work at lower temperatures by use of a proper solvent, i.e., ethyl acetate, benzene, toluene, etc.

The isolation of some of the quaternary ammonium compounds in pure state was frequently attended with difficulty due to their hygroscopic characteristics. Crystallization of some of the compounds was effected through the use of ethyl acetate, acetone, absolute ether, alcohol, and other solvents. Many of the compounds prepared were mixtures i.e., isomeric dichlorbenzylloroldimethyl[2]-ammonium chloride, and no attempt at crystallization was made. It was found suitable, in these cases, to carry the reaction to completion and then to remove the solvent; the residue contained the desired quaternary ammonium compound and was used without further purification.

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[1] The various substituted benzyl chlorides required for the preparation of the compounds were commercially available.
[2] Lorol = mixture of alkyl radicals from C_8H_{17} to $C_{18}H_{37}$ which is obtained from the fatty acids of coconut oil in naturally occurring proportions.

Bacteriological Studies

Phenol Coefficient.—The Food and Drug Administration (F.D.A.) method for determining phenol coefficients (15) was one means of evaluating the quaternary ammonium compounds. *Staphylococcus aureus* (*209*) and *Eberthella typhosa* (Hopkins' strain) were used as the test organisms. The seeding tubes containing the dilutions of the compounds were placed in a water bath at 20° for five minutes, or until the temperature of the bath was reached, before the addition of the test culture. The tubes of F.D.A. broth containing transplants from the seeding tubes were incubated at 37° for forty-eight hours and observed for the presence or absence of visible growth. Tubes showing no growth were tested for bacteriostasis by transferring a quantity of broth mixture to additional tubes containing sterile broth, as outlined in the F.D.A. test procedure.

Bactericidal and Bacteriostatic Tests.—Many of the quaternary ammonium compounds were tested against a variety of bacteria in the presence of a fluid medium. Stock 1% solutions of the compounds were prepared in distilled water. Serial dilutions were made from the stock directly in broth most suited to the organisms to be tested. The drug-broth solutions were distributed in 10-ml. quantities in test tubes and autoclaved at ten pounds for ten minutes. The inoculum in most instances, consisted of 0.2 ml. of a 1:1000 dilution of a twenty-four hour broth-diluted culture of test organism. A number of Gram-negative bacilli representative of the colon-typhoid-dysentery group was tested in the presence of F.D.A. broth. The latter medium was also used for *Proteus*, *Pseudomonas*, and *Vibrio* (*cholora*) cultures. Veal infusion broth (*p*H 7.4)

TABLE I.—PHENOL COEFFICIENTS OF ALKYLDIMETHYLBENZYL-AMMONIUM CHLORIDES AGAINST STAPHYLOCOCCUS AUREUS AND EBERTHELLA TYPHOSA

Alkyldimethyl-Ammonium Chloride (Alkyl Groups)	Benzyl and Substituted Benzyl Groups—Phenol Coefficient Values at 20°							
	Benzyl		4-Nitrobenzyl		3,4-Dichlorbenzyl		Isomeric Dichlorbenzyl	
	S. aureus	E. typhosa	S. aureus	E. typhosa	S. aureus	E. typhosa	S. aureus	E. typhosa
Hexyl (C_6H_{13}-)	<16[a]	<11	<16	<11	<16	<11	<16	<11
Octyl (C_8H_{17}-)	<16	<11	<16	<11	<16	<11	<16	<11
Decyl ($C_{10}H_{21}$-)	50	55	<16	<11	250	200	<16	<11
Dodecyl ($C_{12}H_{25}$-)	214	150	300	300	666	625	714	560
Myristyl ($C_{14}H_{29}$-)	600	450	300	300	833	625	150	120
Cetyl ($C_{16}H_{33}$-)	214	150	76	122	85	60	85	60
Octadecyl ($C_{18}H_{37}$-)	50	66	16	11	16	11	33	11

[a] Figures preceded by < indicate highest concentration of cationic detergent tested, 1:1000, failed to kill test organisms in ten minutes.

Results.—Table I presents the phenol coefficients of the simple benzyl compound and three substituted benzyl derivatives of alkyldimethylbenzyl-ammonium chlorides. The alkyl groups include compounds with carbon chain lengths of C_6 up to and including C_{18}.

From the data given it may be noted that the salts containing carbon lengths of C_6 and C_8 show no germicidal activity when tested by the phenol coefficient procedure. This same negative effect is noted in the 4-nitrobenzyl and isomeric dichlorbenzyl derivatives containing 10 carbons in the alkyl group. The most active compound containing 10 carbon atoms in the alkyl radical is the 3,4-dichlorbenzyl derivative. In practically all instances, an abrupt increase in the phenol coefficients is noted when the carbons in the chain are increased from C_{10} to C_{12}. In some instances a further increase in activity was observed when the carbon atoms were increased to C_{14} (unsubstituted benzyl and 3,4-dichlorbenzyl). However, upon increasing the length of the alkyl group of C_{16} and C_{18}, a gradual reduction in the phenol coefficient values occurred.

Table II presents the phenol coefficients of a series of benzyl derivatives of lorol dimethyl-ammonium chloride. It may be noted that the unsubstituted benzyl derivative has phenol coefficient values of 350 and 333 against *S. aureus* and *E. typhosa*, respectively. Substitution of a chlorine atom in the 2-position in the aromatic (benzyl) nucleus caused a definite reduction of the phenol coefficients. However, the compound containing a chlorine atom in the 4-position gave results which were comparable to the parent unsubstituted quaternary ammonium chloride. Upon increasing the number of chlorine atoms to two or three (trichlorbenzyl) on the aromatic groups, compounds with appreciably higher phenol coefficient values resulted. The 4-nitrobenzyl derivative was somewhat less active than the unsubstituted compound.

TABLE II.—PHENOL COEFFICIENTS OF BENZYL DERIVATIVES OF LOROL[a] DIMETHYL-AMMONIUM CHLORIDES AGAINST STAPHYLOCOCCUS AUREUS AND EBERTHELLA TYPHOSA

Derivatives of Lorol Dimethyl-Ammonium Chloride	Organisms—Phenol Coefficient Values at 20°	
	S. aureus	E. typhosa
Benzyl	350	333
2-Chlorbenzyl	257	266
4-Chlorbenzyl	342	333
2,4-Dichlorbenzyl	600	600
3,4-Dichlorbenzyl	450	300
Isomeric dichlorobenzyl	412	444
Isomeric trichlorbenzyl	571	511
4-Nitrobenzyl	300	300

[a] Lorol = mixtures of alkyl groups from C_8H_{17} up to and including $C_{18}H_{37}$ with average of $C_{12}H_{27}$ (see footnote, page 354).

containing 0.15% dextrose and 0.1% normal horse serum was used in tests in which *S. aureus*, *Diplococcus pneumoniae* Types I, II, and III, and *Streptococcus pyogenes* (#C-203) were studied. Brewer's fluid thioglycollate medium was selected for studying the effects of the quaternary ammonium compounds against the anaerobic clostridia. The inoculum used in the studies on the latter group of bacteria was a 4-mm. loopful of a twenty-four-hour undiluted thioglycollate culture of test organism.

The inoculated media were incubated at 37° and observed for the presence of visible growth at the end of twenty-four, forty-eight, and seventy-two hours. Failure of growth to appear at the initial twenty-four-hour incubation period was considered evidence of bacteriostasis. Tubes showing no growth at the end of seventy-two hours were tested for bactericidal action by transferring three 4-mm. loopfuls of the test mixture to a second tube of liquid medium. Lack of growth in the subculture tubes following seventy-two hours' incubation was taken as evidence of a bactericidal action of the quaternary ammonium compound upon the test organisms in the original broth tube.

Upon increasing the number of carbon atoms in the chain from C_{16} (II-cetyl) to C_{18} (III-octadecyl) an abrupt drop in activity occurs against all Gram-negative bacteria, Gram-positive clostridia, and *S. aureus* cultures. The pneumococci and *S. pyogenes* culture, on the other hand, are as sensitive to the octadecyl as they are to the cetyl or lorol compounds.

The data obtained on the second group of compounds (IV, V, VI), which represent the dodecyl (C_{12}), myristyl (C_{14}), and cetyl (C_{16}) derivatives of 3,4-dichlorbenzyl-ammonium chloride, indicate that an abrupt drop in activity occurs when the carbon chain lengths are increased from C_{14} to C_{16}. The first two compounds give comparable degrees of activity against the entire group of organisms. Concentrations of approximately 1:128,000 are lethal for the typhoid, dysentery, and cholera bacteria, whereas considerably greater concentrations are required to destroy the remaining Gram-negative organisms. The Gram-positive cocci and anaerobic clostridia are uniformly sensitive to the two derivatives in dilutions of 1:200,000 to 1:400,000.

TABLE III.—LIMITING DILUTIONS OF SEVERAL ALKYLDIMETHYLBENZYL-AMMONIUM CHLORIDES SHOWING ANTIBACTERIAL EFFECTS IN NUTRIENT MEDIA

Organism—Strain	Compound—Dilution[a] Bacteriostatic (Bs)—Dilution Bactericidal (Bc) I[b] Bs	I[b] Bc	II Bs	II Bc	III Bs	III Bc	IV Bs	IV Bc	V Bs	V Bc	VI Bs	VI Bc
E. typhosa "H"	256	128	256	128	16	8	128	128	128	128	16	16
E. typhosa "R"	256	128	256	128	16	8	128	128	128	128	16	16
S. dysenteriae "S"	256	256	256	128	32	16	128	128	128	128	8	4
S. dysenteriae "F"	512	256	128	32	32	8	256	128	256	128	16	8
S. dysenteriae "H"	512	256	512	256	64	16	512	256	512	128	64	16
E. coli	64	32	256	128	4	4	128	32	64	16	4	2
A. aerogenes	16	16	32	16	1	<1[c]	32	32	32	16	<1	<1
S. paratyphi	64	32	32	32	4	2	64	32	32	32	1	1
S. enteritidis	32	32	16	16	4	2	32	32	32	32	<1	<1
Proteus vulgaris	16	8	16	16	8	4	16	16	32	16	128	4
Proteus morganii	32	16	128	128	16	16	32	32	64	32	8	4
Ps. aeruginosa "I"	32	32	64	16	1	1	64	64	64	32	<1	<1
Ps. aeruginosa "II"	8	4	1	<1	<1	<1	8	8	1	1	<1	<1
V. cholera "As"	512	256	256	128	64	16	256	64	256	128	4	4
V. cholera "Al"	256	128	256	64	64	16	256	128	256	128	128	64
S. aureus	800	200	400	200	50	50	200	100	800	100	50	25
Pneumo. I	400	100	800	200	400	400	400	200	800	200	200	200
Pneumo. II	200	200	800	400	800	400	800	400	800	200	400	400
Pneumo. III	100	50	200	200	800	800	1600	800	200	100	400	400
S. pyogenes	800	200	1600	400	800	400	800	400	1600	400	800	400
Cl. welchii	200	200	400	400	25	25	800	800	200	200	50	50
Cl. tetani	200	200	400	200	100	25	800	800	400	400	100	50
Cl. histolyticum	200	50	400	400	100	25	800	800	800	200	50	25
Cl. oedematiens	200	200	200	200	25	25	800	800	50	50	50	50

[a] All values given represent dilutions in thousands, i.e., 256 = 256,000.
[b] I = lorol benzyl, II = cetyl benzyl, III = octadecyl benzyl, IV = dodecyl 3,4-dichlorbenzyl, V = myristyl 3,4-dichlorbenzyl, VI = cetyl 3,4-dichlorbenzyl.
[c] Figures preceded by < indicate the highest concentration of cationic detergent tested failed either to kill or inhibit the growth of the test organisms.

Results.—Table III presents typical results obtained upon using three representative compounds in two of the series of alkyldimethylbenzyl-ammonium chlorides against bacteria in the presence of nutrient media. In reviewing the data of the first three compounds (I, II, III), which represent the lorol, cetyl, and octadecyl derivatives with no substitution in the benzyl groups, it will be noted that C_{12} and C_{14} derivatives give results comparable to those obtained with the lorol and cetyl derivatives of the unsubstituted alkyldimethylbenzyl-ammonium chlorides (I, II). Furthermore, there is a close correlation in the degrees of activity of the octadecyl (C_{18}) derivative of the latter group of compounds and the cetyl analog of 3,4-dichlorbenzyl-ammonium chloride.

Surface Tension.—The surface tensions of 1% aqueous solutions of the quaternary ammonium salts given in Table I were determined by the ring method with a du Nouy tensiometer. Readings made soon after the solutions were prepared and again after standing at room temperature for one week indicated that the initial values remained constant. The values, corrected for temperature, are presented in Table IV.

the germicidal portion of the molecule is toward the outside. This is offered as the explanation for the presence of viable, unharmed organisms on the skin under the "imperceptible" film (17). Additional studies are in progress to determine the relationship of surface and interfacial tensions of homologous series of quaternary ammonium germicides and the mechanism of action of the derivatives upon microorganisms.

TABLE IV.—SURFACE TENSION OF 1% AQUEOUS SOLUTIONS OF ALKYLDIMETHYLBENZYL-AMMONIUM CHLORIDES

Alkyldimethyl-Ammonium Chloride (Alkyl Groups)	Benzyl and Substituted Benzyl Groups—Surface Tension—Dynes/Cm.			
	Benzyl	4-Nitrobenzyl	3,4-Dichlorbenzyl	Isomeric Dichlorbenzyl
Hexyl (C_6H_{13}-)	60.3	57.0	49.3	55.1
Octyl (C_8H_{17}-)	50.1	49.0	40.4	45.4
Decyl ($C_{10}H_{21}$-)	40.8	39.5	31.6	33.8
Dodecyl ($C_{12}H_{25}$-)	38.9	40.5	31.8	34.2
Myristyl ($C_{14}H_{29}$-)	37.2	39.6	33.6	33.0
Cetyl ($C_{16}H_{33}$-)	35.4	38.7	32.6	33.4
Octadecyl ($C_{18}H_{37}$-)	36.6	38.1	34.0	34.5
Lorol[a]	34.5	40.5	29.0	30.6

[a] See legend under Table II.

Results.—From the data given in the table, it may be noted that a drop of approximately 10 dynes per centimeter is obtained on solutions prepared from the salts containing carbon chain lengths of C_8 as compared to C_6. A corresponding reduction in surface tension is obtained when the chain length is increased from C_8 to C_{10}. Upon increasing the number of carbon atoms in the chain to C_{12}, C_{14}, C_{16}, and C_{18}, however, no significant change in surface tension occurred. In other words, a plateau is reached with the 1% quaternary ammonium salt solution with the introduction of 10 carbon atoms in the alkyl chain, beyond which further addition of carbon atoms does not influence the surface tension of the solution. It is apparent, therefore, that there is little, if any, correlation between surface tension and degree of antibacterial activity of the compounds with carbon chain lengths of C_{12} up to and including C_{18}.

An attempt was made to determine the interfacial tension between aqueous solutions of the homologous series of the quaternary ammonium derivatives and an oil phase. This study revealed that by using corn oil and mineral oil a reading on the du Nouy interfacial tensiometer could be obtained only with the C_6 compounds (12–14 dynes/cm.). The solutions prepared with the C_8, and the remaining long-chain alkyl compounds, failed to show any evidence of tension at the water-oil interface. Rahn (16) has demonstrated that quaternary ammonium germicides are strongly adsorbed on the surfaces of organisms. He postulated, moreover, that a film is formed on hands submersed in a solution of a quaternary ammonium compound. This film is believed to be oriented with the inactive organic end of the molecule or ions directed toward the skin, whereas

SUMMARY

The bactericidal and bacteriostatic properties of homologous series of benzyl quaternary ammonium compounds having substituents in the aromatic nucleus are described. Salts containing carbon chain lengths in the alkyl group of C_6, C_8, and in some instances C_{10}, show no germicidal activity in concentrations as high as 0.1 per cent (1:1000) when tested by the phenol coefficient procedure. An abrupt increase in the phenol coefficient occurs when the carbons in the straight chain are increased from C_{10} to C_{12}. With some of the compounds a further increase in activity was noted when the carbon atoms were increased to C_{14}. However, an abrupt drop in phenol coefficient occurred upon increasing the length of the alkyl group to C_{16} and C_{18}.

The substitution of one or two chlorine atoms on the aromatic nucleus and the resulting effects on the phenol coefficient values are described. In general, the quaternary ammonium salts containing two or three chlorine atoms on the benzyl radical are more active than those compounds containing one halogen atom or none.

Several of the compounds were tested in high dilutions against bacteria in the pres-

ence of nutrient media. The critical zone of maximum activity with an abrupt drop in antibacterial action occurs in the compounds containing C_{14} to C_{16} or C_{16} to C_{18}, respectively. The Gram-positive cocci and anaerobic clostridia are destroyed by some of the compounds in concentrations of 1:200,000 to 1:400,000 in broth. The Gram-negative colon-typhoid-dysentery and related groups of organisms are considerably more resistant than the Gram-positive bacilli to the antibacterial action of the quaternary ammonium salts.

Surface-tension studies on homologous series of several compounds indicate that a decrease or lowering of approximately 10 dynes per cm. occurs upon increasing the number of carbon atoms in the alkyl group from C_6 to C_8 and again from C_8 to C_{10}. No appreciable change in surface-tension values occurs upon further increase in the chain lengths to C_{12}, C_{14}, C_{16}, and C_{18}. There is no apparent correlation between surface tension and degree of antibacterial activity of a homologous series of the quaternary ammonium salts containing 12 and up to and including 18 carbon atoms in the straight chain alkyl group.

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Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting specific identification of the work to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to the Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, Dr. Robert P. Fischelis, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1948 Award, nominations must be received before January 1.

Establishment of the new iodine award was announced at the Milwaukee convention of the AMERICAN PHARMACEUTICAL ASSOCIATION, which will administer the competition.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During a period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Iodine Educational Bureau, Inc.

The award consists of $1000 and a diploma setting forth the reasons for selection of the recipient. It may be presented biennially at the annual meeting of the AMERICAN PHARMACEUTICAL ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL of the AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION's Council and which functions under prescribed rules.

Should the award committee fail to find a suitable recipient in any biennium, two awards may be made during the next biennium in the same or successive years, if suitable recipients are selected.

Studies on Alcohol-Soluble Fungistatic and Fungicidal Compounds. II. Evaluation of a Fungicidal Laboratory Test Method*,†

By MILTON J. GOLDEN and KURT A. OSTER‡

There exists an urgent need for the establishment of a laboratory test method for the assay of water-insoluble fungicidal compounds which is both reproducible and meaningful. This study presents a critical evaluation of the existing procedures and a newly developed *in vitro* method for the evaluation of alcohol-soluble fungicides. The fungicidal activities of ten compounds commonly used in the treatment of foot dermatophytosis are compared, employing the new modified method.

THE STATEMENT "The human foot has become the target of unbelievable abuse," made recently by Underwood, *et al.* (1), aptly describes the confused status of the chemotherapy for dermatophytosis of the foot. The necessity for accurate laboratory and clinical examination and re-examination of the multitude of compounds now used for the treatment of this condition cannot be overemphasized.

A recent paper (2) was concerned with a discussion of and suggested modifications to the existing test methods for the demonstration of fungistasis along with an investigation of the fungistatic action of ten alcohol-soluble compounds commonly employed in the treatment of dermatophytosis. The present study aims at a similar clarification and modification of fungicidal test methods.

Much of the present confusion regarding the tests for substances used for dermatophytosis may be attributed to the loose usage of the terms *fungistatic* and *fungicidal*. We understand *fungistatic* action of a substance to indicate its growth-preventing effect on a culture of fungi so long as it is in contact with the organisms. *Fungicidal* action, on the other hand, designates the killing effect of a compound on a fungus culture after contact for a limited period of time, making certain that the compound is completely removed from the culture following the contact time and that the basic structure of the colony has not been altered either by the compound or by the test method.

This definition would then exclude, as far as the test methods are concerned, all compounds which form permanent complexes with the fungus proteins, such as various metal salts, and those which destroy the fungus itself, as strong acids or bases. Likewise, all fungicidal methods which alter the basic structure of the colony would be excluded by this definition, thereby eliminating the method suggested by Schamberg and Kolmer (3), where the matted culture is broken up artificially and also the one suggested by Emmons (4) which makes use only of a microconidial suspension. The latter method would not be useful for testing alcohol-soluble compounds.

There remains then the fungicidal test method suggested by Burlingame and Reddish (5), which avoids any alteration of the basic structure of the fungus colony, but which possesses other shortcomings, especially when studying water-insoluble fungicides. The adaptation and improvement of this latter method for use in testing alcohol-soluble compounds has been made the subject of this report.

EXPERIMENTAL

Burlingame and Reddish Method (5).—Petri dishes of Sabouraud's agar are inoculated with a culture of the test organisms and incubated at room temperature for five days, at which time the cultures are cut into 1-cm. squares or disks 1 cm. in diameter.

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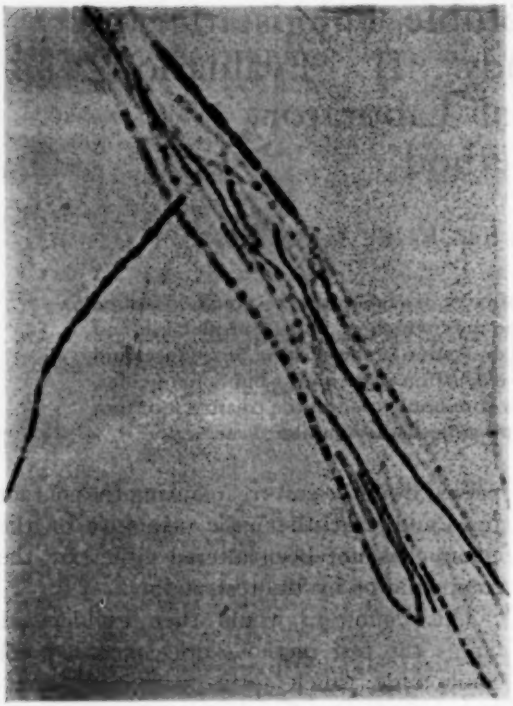

Fig. 1.—Five-day-old culture with hyphal structure predominating.

The surface of each culture is flooded with 15 cc. or more of the test fungicide. After five, fifteen, and thirty minutes, one of the squares or disks of culture and agar is removed from each plate and placed in 10 cc. of sterile broth. Shaking the broth tube lightly for five minutes serves to wash out the excess fungicide from the matted culture. The culture block is removed from the broth and spread, culture side down, over the surface of a sterile slant of Sabouraud's agar, which is then incubated at room temperature for three weeks and observed for growth. An effective fungicide should kill the test organism within five minutes' contact.

Careful scrutiny of the steps involved in performing this technique reveals the following shortcomings, most evident when alcohol-soluble fungicides are employed:

1 The test culture is not sufficiently aged.

2. The contact time of the fungicide with the culture is overly long, and no serum has been incorporated in the medium to detect any eventual protein effect.

3. A water-insoluble, alcohol-soluble fungicide cannot be removed from the culture disk by shaking it in watery broth.

Age of Culture.—Microscopic examination of cultures of *Trichophyton mentagrophytes* (#640 Emmons) reveals that hyphal structure predominates in a five-day-old culture. After ten days an abundance of microspores or microconidia can be observed, while a fifteen-day-old culture exhibits many thick-walled chlamydospores. These time variations in the basic culture characteristics, as demonstrated in Figs. 1, 2, and 3, should be afforded careful consideration in any fungicidal test method. Since these test conditions should most closely simulate the human pathological picture found in dermatophytosis, the test culture to be used should contain most of the various morphologic structures of fungus growth and reproduction. Use of the relatively immature five-day-old culture does not fulfill this need. A similar idea has been propounded by McCrea (6).

Length of Contact Time with Fungicide and Absence of Proteins in Culture Medium.—Burlingame and Reddish (5) suggest contact of the culture with the test fungicide for five, fifteen, and thirty minutes. However, such long exposures are not practical when alcohol-soluble, water-insoluble fungicides are being tested. A 95% alcohol control was fungicidal in itself after ten minutes' contact with a five-day-old culture. In order to make the test conditions more stringent and the results more meaningful the contact time for alcohol-soluble fungicides has been reduced by us to one minute.

Proteins have been found to detract from the efficacy of fungicides. It was for this reason that the authors have incorporated horse serum in the Sabouraud's medium in a concentration of 10%. This inclusion effected a considerable diminution of the activity of some compounds.

Fig. 2.—Ten-day-old culture with occurrence of microspores.

Washing Step.—Once again the Burlingame and Reddish technique (5) is not applicable to alcohol-soluble compounds. Logically, a water-insoluble substance cannot be removed from the test culture disk by shaking in a watery broth solution. This could be demonstrated by a simple test. A 1% solution of salicylic acid in 95% ethyl alcohol was found to be fungicidal on five minutes' contact with a five-day-old culture, when tested by the original Burlingame and Reddish method, i.e., observing the washing step in aqueous broth. That a considerable amount of salicylic acid adhered to such a culture disk *after* the broth washing step was proved by a positive ferric chloride test. However, when a similar salicylic acid treated culture disk was washed for five minutes in a 30% acetone-water mixture, the substance was properly removed, as indicated by an almost negative ferric chloride reaction, and the final test results showed that a 1% salicylic acid solution is *not* fungicidal. At best the Burlingame and Reddish technique demonstrates a continued fungistatic rather than fungicidal action of water-insoluble compounds.

It was ascertained by us that a 30% acetone-water mixture suffices in most cases to remove the adhering fungicide and that this mixture is not fungicidal in itself even after a fifteen-minute contact with a five-day-old fungus culture.

Modified Test Method for the Evaluation of Alcohol-Soluble Fungicides.—Taking all of the described findings into consideration, the adoption of the following method gave reproducible and significant results with alcohol-soluble fungicides.

Petri dishes of Sabouraud's agar containing 10% horse serum are streaked with a culture of *T. mentagrophytes* (#640 Emmons) and permitted to grow at 28° ± 1° for five, ten, fifteen, and twenty days, respectively. On the day of the test the cultures are cut into disks of 1 cm. diameter with a sterile cork borer and transferred with aseptic precautions to seeding tubes containing 10 cc. of the various concentrations of the test fungicide in 95% ethyl alcohol. After a one-minute contact with the fungicide the disk is transferred to 10 cc. of sterile broth and shaken lightly for three minutes to free the matted culture of any water-soluble or miscible material. The culture block is then removed from the broth and washed in 10 cc. of 30% acetone-water mixture for five minutes, thereby removing the fungicide adhering to the mycelium. Following this acetone washing step, the disk is once more immersed in sterile broth for two minutes to remove possible traces of acetone and then spread, culture side down, over the surface of a sterile slant of Sabouraud's agar. These final slants are incubated at 28° ± 1° for three weeks and observed for growth.

This test method calls for the immersion of the individual culture-agar disks in tubes containing the alcoholic fungicide in various dilutions, rather than the procedure of pouring the fungicide over the intact culture surface before cutting disks.

Fig. 3.—Fifteen-day-old culture showing presence of large chlamydospores.

This modification is time and material saving and does not impair the accuracy of the results.

Fungicidal Evaluation of Ten Alcohol-Soluble Compounds.—The ten alcohol-soluble compounds previously subjected to studies on test methods for fungistatic activity were reinvestigated, this time with respect to their fungicidal evaluation, employing the modified fungicidal test method described above. They were phenol, resorcinol, salicylic acid, benzoic acid, cresol U. S. P., metacresylacetate, thymol, chlorthymol, propionic acid, and undecylenic acid.[1] To determine the minimal fungicidal concentration of each of the ten compounds, three separate tests were carried out for every dilution of each compound on five-, ten-, fifteen-, and twenty-day-old cultures of *T. mentagrophytes*. The critical fungicidal concentration was established when at least two of three parallel tests showed no growth after three weeks' incubation. The results obtained are shown in Table I. The compounds have been arranged in an order of decreasing activity, with chlorthymol the most potent and resorcinol the least potent. The end points for cresol and metacresylacetate were not as sharply defined and reproducible as the other substances. With the remaining eight compounds fungicidal activity was established within limits of 0.1, 0.5, or 1.0% variation in concentration.

[1] Undecylenic acid supplied by Dr. L. Reiner, Wallace and Tiernan Products, Inc., Belleville, N. J.

TABLE I.—CRITICAL FUNGICIDAL CONCENTRATIONS OF TEN COMPOUNDS DISSOLVED IN 95% ALCOHOL AS DETERMINED WITH CULTURES OF TRICHOPHYTON MENTAGROPHYTES (#640 EMMONS) AT VARIOUS STAGES OF GROWTH

Compound in 95% Ethyl Alcohol	Minimal Fungicidal Concentration Age of Test Culture in Days 5	10	15	20
Chlorthymol	0.1%	0.5%	0.5%	0.5%
Thymol	1.5%	2.0%	2.0%	3.0%
Undecylenic acid	1.25%	2.5%	2.5%	2.5%
Salicylic acid	2.0%	7.0%	8.0%	9.0%
Benzoic acid	2.25%	8.0%	9.0%	9.0%
Cresol U. S. P.	6.0%	11.0%	11.0%	10.0%
Metacresylacetate	6.0%	12.0%	14.0%	13.0%
Propionic acid	8.0%	>15.0%	>15.0%	>15.0%
Phenol	9.0%	15.0%	>15.0%	>15.0%
Resorcinol	>15.0%	>15.0%	>15.0%	>15.0%

DISCUSSION

An examination of the data presented in Table I reveals a sharp rise from the fungicidal values obtained with five-day-old fungus cultures to those of ten-day-old cultures, supporting McCrea's contention that a five-day-old fungus culture is not sufficiently mature for the critical testing of fungicides (6). Findings with ten-, fifteen-, and twenty-day-old cultures show very slight differences. The use of a fifteen-day-old culture, containing, as it does, hyphae, microconidia, and chlamydospores, along with use of the one-minute contact time, would impose highest requirements on a new fungicide. The authors therefore suggest this culture age as the basis for any future tests.

The final fungicidal concentrations of propionic acid, phenol, and resorcinol were not tested for, since concentrations as high as 15% were still not fungicidal, an observation which may serve to eliminate these substances from the group designated as fungicides. Arranging the ten investigated compounds in an order of decreasing fungicidal activity, one finds that the sequence does not correspond to that encountered when the same compounds are similarly arranged for fungistatic activity. The fungistatic sequence was presented in a previous study (2).

In actual therapy, fungicidal compounds are not usually dispensed in a solution of 95% alcohol. We have noted that diluting down this solvent diminishes considerably the fungicidal activity of any compound dissolved therein. For this reason the data given here are valid only for the conditions stated and should be regarded merely as an expression of the relative activity of the tested compounds. A new dilution must be subjected to new tests and would furnish different values.

The fungicidal activities of the substances under test were found to be relatively low with the exception of chlorthymol, which, however, exhibits strong skin-irritating properties (7). It would seem worth while, therefore, to employ these new test methods, both fungistatic (2) and fungicidal, in a search for more powerful substances and to eliminate weak and inefficient compounds from the medical armamentarium.

SUMMARY

1. The Burlingame and Reddish technique for demonstrating fungicidal activity has been modified and adapted for the assay of alcohol-soluble fungicides.

2. Reasons for the suggested test method changes are presented and discussed. The authors propose the use of a fifteen-day-old fungus culture, the observance of a culture-fungicide contact time of one minute, the incorporation of serum in the culture medium, and the substitution of an acetone-water mixture for the aqueous broth employed in the washing phase of the test procedure.

3. The relative fungicidal activities of ten commonly used alcohol-soluble fungicides have been evaluated, employing the modified test method, and the results discussed.

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The Assay of Digitalis. V. The Guinea-Pig Method*

By H. A. BRAUN and A. SIEGFRIED†

Five preparations of digitalis have been assayed by the guinea-pig method and have been compared with the U. S. P. XI one-hour frog method and the U. S. P. XII intravenous cat method. Four of the five preparations show good agreement in the potency estimates between the cat and guinea-pig methods. The potency of the fifth assayed by the guinea-pig method yielded a result differing by more than 20% from that obtained by the intravenous cat method.

ONE OF THE problems encountered in the field of biological standardization is the determination of the proper method of assay and the selection of the most adequate test animal.

The U. S. P. XII intravenous cat method has been generally considered satisfactory in that it yields almost identical results in the various laboratories where it is utilized (1) and the results obtained are in general agreement with the assay on humans. However, since the adoption of the U. S. P. XII method for assaying digitalis, it has become evident that cats are often difficult to obtain and that their cost is considerable. We were led to investigate the practicability of restudying the guinea-pig method of Knaffl-Lenz (2, 3) for several reasons. Guinea pigs are usually quite available and considerably less costly than cats. It would seem that this species should be more homogeneous than the cat since the conditions under which they are bred are relatively constant and there would scarcely seem to be any cause for variation other than that inherent in the test animal itself. The method of Knaffl-Lenz has found widespread use as a method of biological assay and has been officially adopted by several European and South American countries. To date there does not seem to exist any detailed investigation of certain phases of this bio-assay especially those defining the conditions and variables based on a sufficiently large number of animals.

We have investigated some of the salient features of this assay and have evaluated the various factors statistically in order to define the conditions necessary to obtain greater accuracy in this method. Seven hundred and twenty-five guinea pigs were used in the investigation of the various phases of this assay.

EXPERIMENTAL

In performing this assay the method used was generally identical with the Hatcher–Brody cat method (4) as modified by Magnus (5). The guinea pigs were prepared for infusion approximately one hour after injection of the fixed dose of anesthetic or immediately after induction in the case of ether. The animals were tied on suitable boards and the brachial vein just below the axilla was exposed. The properly diluted tincture was transferred to a 10-cc. microburette to which was attached by means of a rubber tube a 1-inch 26-gauge hypodermic needle. The needle was inserted into the brachial vein and tied into place. The rate of injection in all our experiments was kept constant at 0.2 cc. per minute. The constant flow from the burette was regulated by a Dale and Burn constant infusion apparatus. Arrest of the heart was determined by palpation and auscultation of the chest. Toward the end of the experiment after the heart had stopped, natural respiratory movements sometimes reappeared and their cessation and slight convulsions confirmed final cardiac arrest. The chest was opened and the condition of the heart and the abdominal organs was noted.

Preparation of the Digitalis Tinctures.—Tinctures used for the infusion were prepared from the powdered digitalis by the U. S. P. XII method using 0.1 Gm. of powdered leaf per cc. of U. S. P. XII Digitalis Menstruum. The proper dilutions of the macerate for injection into the animal were made with isotonic solution of sodium chloride. Unless otherwise stated the tincture which was used in all the present experiments was prepared from the U. S. P. Reference Digitalis Powder.

SALIENT FEATURES

Size.—In the original paper on this method Knaffl-Lenz (2) recommended that guinea pigs weighing between 500 and 800 Gm. should be used for the assay. He stated that animals less than 500 Gm. seemed more resistant to digitalis while animals

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weighing over 800 Gm. were less so and less of the drug was required to cause death. Goldberg (8) suggested the use of guinea pigs having a range of 350 to 500 Gm. and stated that the variation of animals within a single group should be from 50 to 75 Gm.

Customarily, the results of the digitalis assay would be expressed in mg. of digitalis necessary to produce death per 100 Gm. of body weight of guinea pig. Early in our investigations we, too, noted that when the quotient (mg. of digitalis)/(100 Gm. body wt.) Formula A was used to express the lethal dose of digitalis for guinea pigs ranging in weight from 196 to 893 Gm., a series of values showing great variations were obtained. The results were high for light guinea pigs and low for heavy animals. This condition is illustrated in Fig. 1.

Accordingly, the question arose as to why it can be assumed that a direct proportionality exists between body weight and fatal dose. It is possible that there is a more valid relationship. A standard statistical procedure was applied to the problem and the calculations showed that the ratio of the fatal dose to the body weight can be adjusted to allow for any sensitivity due to variations in the body weight. The adjustment for the body weight is called the size factor and the lethal dose of digitalis for the guinea pig under present experimental conditions has been found to equal 0.64 ± 0.039. In other words, the assay results can be more accurately expressed by Formula B (mg. of digitalis)/(100 Gm. body wt.)$^{0.64}$. The effect of variations of body weights on the observed lethal dose when animals of the same series are divided into light and heavy groups is illustrated below.

Heavy vs. Light Guinea Pigs—

U. S. P. Digitalis Reference Powder = 1:30 dilution
No. of animals = 101
Wt. range = 196 to 893 Gm.
Av. wt. = 512 Gm.

Divided Into Two Groups:—

	Light	Heavy
Wt. range	196–543	547–893
Av. wt.	354	673
No. of animals	51	50

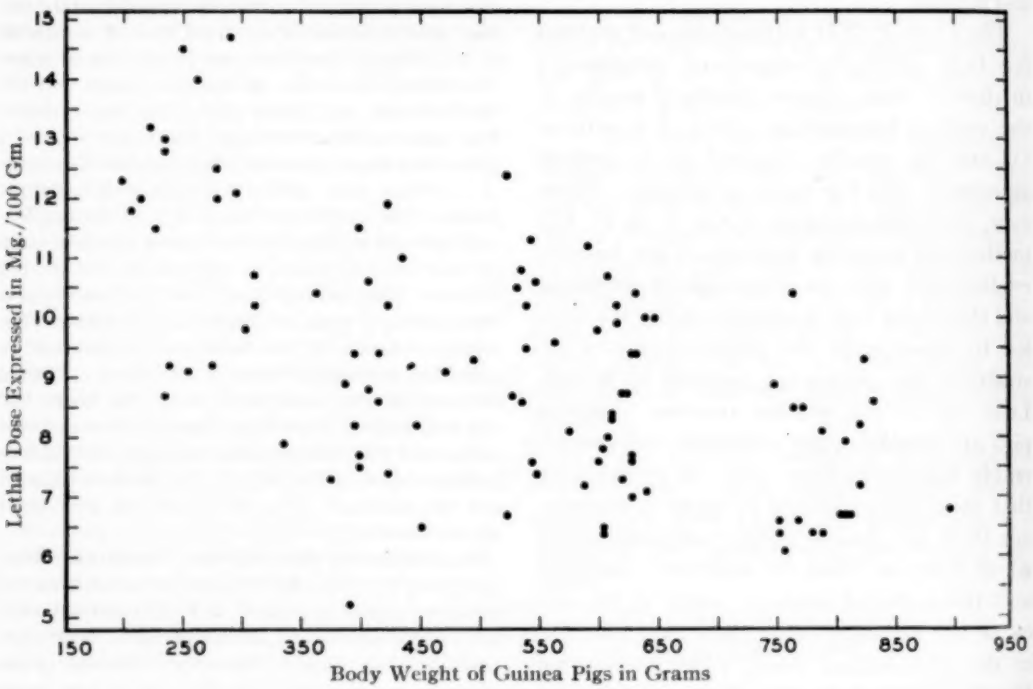

Figure 1.

TABLE I.—A COMPARISON OF THE SUSCEPTIBILITY OF GUINEA PIGS FROM VARIOUS SOURCES

Source	Wt. Range Av. Wt.	Dilution	Sex	No. of Animals	Lethal Dose, Mg./100 Gm.	Adjusted Lethal Dose Mg./(100 Gm.)$^{0.64}$	Potency[a]	Std. Error
A	360–820 599	1:30	M	58	8.5 ± 0.21	16.2 ± 0.37	Std.	.....
B	196–893 438	1:30	M	22	9.7 ± 0.51	16.5 ± 0.51	97.8%	±4.3%
C	207–422 309	1:30	M	21	9.7 ± 0.57	14.5 ± 0.70	111.2%	±8.1%

[a] In terms of Source A.

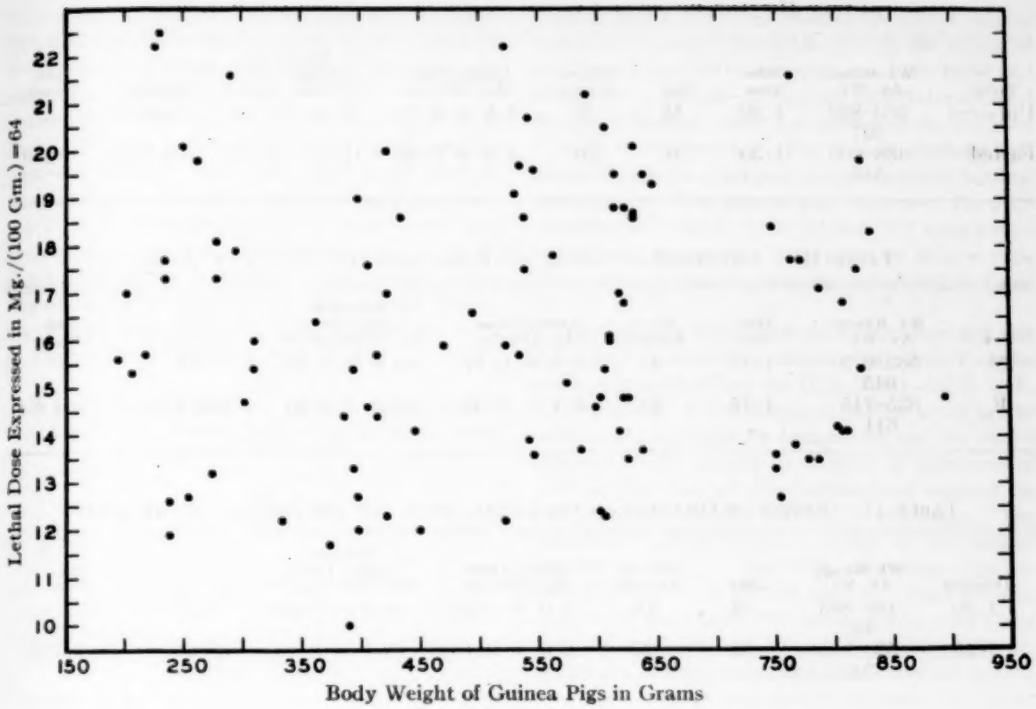

Figure 2.

Formula A			
Lethal dose	9.9 mg./100 Gm.	122.4%	8.1 mg./100 Gm.
Std. error	±0.32 mg./100 Gm.	±8%	±0.20 mg./100 Gm.
Formula B			
Adjusted lethal dose	15.7 mg./(100 Gm.)$^{0.64}$	97.2%	16.1 mg./(100 Gm.)$^{0.64}$
Std. error	±0.45 mg./(100 Gm.)$^{0.64}$	±6%	±0.35 mg./(100 Gm.)$^{0.64}$

The difference is significant when Formula A is utilized.

The effect of using Formula B is evident in Fig. 2. It is apparent that there is a much greater uniformity of response. By using Formula B there is no need for specifying a range of body weights in the guinea pigs.

The figure 0.64 ± 0.039 was calculated from the results obtained on 286 guinea pigs. No series has yet been found which possessed a size factor differing significantly from this value. The size factor 0.64 has been used consistently as the basis for comparison of all series in this paper. It is particularly important when extreme weight ranges are involved. The determination of the size factor has previously been utilized by Bliss (6) and by Dawson (7).

Influence of Source of Supply.—Goldberg (8) has stated that one of the prerequisites for obtaining efficient bioassay with guinea pigs is that the animals must come from homogeneous stock. We have in the course of our investigations studied the variability of guinea pigs from three different sources of supply. In all 101 male guinea pigs weighing between 196 and 893 Gm. were used for this experiment. It was our policy to use guinea pigs only after they had been in the laboratory on an adequate diet for at least one week. The results are tabulated below.

From Table I it can be seen that the mean lethal dose of digitalis for the guinea pigs from three different sources is not significantly different and the guinea pigs can therefore be considered homogeneous.

Influence of Fasting the Guinea Pigs.—In his original version of the bioassay of digitalis, Knaffl-Lenz (2) specifies that the animals should be fasted for six to eight hours prior to the assay. Twenty male guinea pigs were selected to determine whether fasting had an important influence on the results of the assay. The guinea pigs which were fasted for twelve to eighteen hours were compared with a series of unfasted animals. The data obtained in this experiment are listed in Table II. It is evident from Table II that fasted and unfasted guinea pigs do not differ significantly, and unfasted animals can be utilized for this assay.

Influence of Sex.—The influence of sex on this biological assay has not been reported. To get a better understanding of this factor, two series of animals from the same source of supply consisting of 24 males and 20 nonpregnant females were compared. The results of this experiment are summarized in Table III. These data show that there

TABLE II.—INFLUENCE OF FASTING ON THE LETHAL DOSE AND POTENCY

Series	Wt. Range Av. Wt.	Dilution	Sex	No. of Animals	Lethal Dose, Mg./100 Gm.	Adjusted Lethal Dose, Mg./(100 Gm.)$^{0.61}$	Potency	Std. Error
Unfasted	360–820 597	1:30	M	58	8.5 ± 0.21	16.2 ± 0.37	Std.	...
Fasted	488–603 546	1:30	M	20	8.4 ± 0.40	15.4 ± 0.72	104.7%	±5.0

TABLE III.—INFLUENCE OF SEX ON THE RESULTS OF THE GUINEA PIG ASSAY

Sex	Wt. Range Av. Wt.	Dilution	No. of Animals	Lethal Dose, Mg./100 Gm.	Adjusted Lethal Dose, Mg./(100 Gm.)$^{0.61}$	Potency	Std. Error
M	532–975 645	1:15	24	8.4 ± 0.27	16.4 ± 0.37	Std.	...
F	535–715 611	1:15	20	8.1 ± 0.34	15.5 ± 0.60	105.9%	±4.6

TABLE IV.—EFFECT OF DILUTION ON THE LETHAL DOSE AND THE POTENCY OF THE ASSAY

Dilution	Wt. Range Av. Wt.	Sex	No. of Animals	Lethal Dose, Mg./100 Gm.	Adjusted Lethal Dose, Mg./(100 Gm.)$^{0.61}$	Potency	Std. Error
1:30	196–893 487	M	101	9.0 ± 0.21	15.9 ± 0.29	Std.	.....
1:15	528–975 703	M	55	8.0 ± 0.16	16.1 ± 0.26	98.5%	±2.7%

is no significant difference in the susceptibility of male and female guinea pigs to digitalis.

Influence of the Concentration of the Solution and Duration of the Assay.—The rate of injection in all our experiments was kept constant at 0.2 cc. per minute. However, the concentration of the diluted tincture was varied. Knaffl-Lenz (2) showed that when his results were calculated on the basis of powdered digitalis leaf the quantity required to cause death was greater for a 0.5% solution than for a 1 to 2.5% solution. Otterström (9) and Levy and Otterström (10), on the other hand, were unable to produce any change in the lethal dose when the concentration was varied. In the course of their experiments they found that the standard error of the experiment was less when the concentration was adjusted so that the assay lasted approximately fifteen minutes and that it rose with increased duration of thirty to eighty minutes. Brun (11) varied the length of the experiment from fifteen to fifty-seven minutes and found no change in the lethal dose. Goldberg (8) found that the lethal dose or log lethal dose varied proportionately to the quantity of drug injected per minute per 100 Gm. of body weight. In our investigations two dilutions were used, *viz.*, a 1:15 and 1:30.

The time factor or duration of the experiment is undoubtedly of great importance not only for the guinea-pig method but also for other intravenous methods. In the guinea-pig assay the average length of the assay for a 1:15 dilution was forty-one minutes, while the animals injected with a 1:30 dilution averaged seventy-two minutes. These average times are in keeping with those originally recommended for the cat method by Hatcher and Brody (4).

The data summarized in Table IV demonstrate that there is no statistically significant difference in the adjusted lethal doses and the potency when a comparative study of the 2 dilutions is made. In view of these results the dilution 1:15 was employed in most of the experiments since it was less time consuming, and appeared also to give somewhat less variable results.

Influence of the Anesthetic.—In his original directions for the guinea-pig assay, Knaffl-Lenz (2) stated that the animals must be anesthetized with ether. Later investigations by Knaffl-Lenz (3) and Gaddum (12) indicated that ether caused large deviations in the lethal doses and these workers recommended the use of 1 Gm. of urethane per Kg. in a 25% solution. In some of our preliminary experiments when ether was used it became apparent that the adjusted lethal doses were too variable to be of value for routine assays.

Three other anesthetics were administered intraperitoneally, *viz.*, sodium phenobarbital 100 mg./Kg. in aqueous solution, urethane 1 Gm./Kg. in aqueous solution, or chloretone 175 mg./Kg. in 50% ethyl alcohol. During the course of our investigation in which we studied the influence of the anesthetic on the adjusted lethal dose and on the relative potency of three digitalis powders, it became evident that sodium phenobarbital was the anesthetic of choice for these experiments. Sodium phenobarbital gave adjusted lethal doses comparable to

those obtained with ether but not nearly as variable. Urethane and chloretone generally yielded adjusted lethal doses which were smaller than those obtained with ether and phenobarbital.

The effect of anesthetics on the potency of three digitalis powders is shown in Table V. Thirty guinea pigs were used for each anesthetic, 10 for each of three powders. The three powders compared were the U. S. P. Digitalis Reference Powder, the 1936 International Standard, and the so-called U. S. P. Study Powder, upon which a collaborative assay was conducted in 1940–1941. This powder following dilution with exhausted marc became the U. S. P. Digitalis Reference Standard (1942).

TABLE V.—THE ADJUSTED LETHAL DOSES MG./(100 GM.)$^{0.64}$ OF THREE DIGITALIS POWDERS WITH VARIOUS ANESTHETICS

	Ether	Sodium Phenobarbital	Urethane	Chloretone
U. S. P. Study Powder	19.1	22.1	15.1	14.6
1936 International Std.	15.7	17.9	13.6	14.7
U. S. P. Digitalis Pwd.	15.4	16.7	11.1	11.9

York Heart Association Powder No. 7. C was a tablet submitted to the laboratory in the course of regular activity in the enforcement of the Food and Drugs Act. Sample D was the 1936 International Standard Powder, while E represented the so-called U. S. P. Study Powder. The U. S. P. Digitalis Reference Powder was utilized as the standard in these comparisons. Ten guinea pigs were used for each preparation. The U. S. P. XI and XII assays were carried on concurrently with those on guinea pigs. The results of these experiments are shown in Table VI.

It is apparent from these data that there is a fair agreement between the estimates of potency of the guinea-pig method and the U. S. P. XI and U. S. P. XII methods. If the values of the cat assay are to serve as the standard for comparison and the stated potency of the sample is allowed a tolerance of ±20%, then four of five preparations assayed by the guinea-pig method will fall in this range. The exception is Preparation B, which on two assays yielded results more than 20% lower than that obtained by the U. S. P. XII method. Recently Allmark and Bachinski (18) have shown that there were differences in potency of 25% or more between the results of the guinea-pig and cat method in 50%

TABLE VI.—COMPARISON OF THE POTENCY OF VARIOUS DIGITALIS PREPARATIONS ON GUINEA PIGS, FROGS AND CATS

Preparation	Guinea-Pig Assay: Ether as Anesthetic, %	Guinea-Pig Assay: Na Phenobarbital as Anesthetic, %	U. S. P. XI One-Hr. Frog Method, %	U. S. P. XII Cat Method, %
A	91.6 ± 8.6	102.0 ± 8.0	104 ± 4.0	95.3 ± 4.8
B	62.6 ± 5.9	67.5 ± 8.5	57 ± 5.0	84.9 ± 4.4
C	56.2 ± 4.7	63.5 ± 6.4	44 ± 3.0	67.8 ± 5.6
D	131.7 ± 6.7	125.0 ± 6.8	106 ± 3.0	113.0 ± 4.6
E	108.3 ± 8.2	101.5 ± 6.7	106 ± 7.0	114.0 ± 5.3

Accuracy of the Method.—The accuracy of the guinea-pig method has varied somewhat in the hands of different investigators. Knaffl-Lenz (3), Trevan, Boock, Burn, and Gaddum (13), Otterström (9), Gram (14), Marri and Ciappi (15), Brun (11), Straub, Kanna, and Zinnitz (16), Goldberg (8), and Eichbaum (17) have found that the coefficient of variation was in the order of 10% to 15%. From the data obtained in assaying 101 guinea pigs using a 1:15 dilution of the tincture the coefficient of variation from our experiment was determined as 13.8%. Using a 1:30 dilution on 206 guinea pigs the coefficient of variation was found to be 17.4%. A coefficient of variation of 13.03% for cats was reported by de Lind van Wijngaarden (5).

Comparison of Assay Results of the Guinea-Pig Method with the U. S. P. XI Frog and U. S. P. XII Cat Assay.—The potency of five digitalis preparations was determined by the three methods. Of the five preparations examined, four were samples of powdered digitalis, while the fifth was a tablet. Preparation A was a powdered leaf assayed at the time of importation from England. B was the New

of their assays. This behavior of certain preparations may be indicative of a fallacy inherent in this method. The low standard errors obtained with ether in our series of assays were unusual and quite contrary to our previous extensive experience with this anesthetic.

DISCUSSION

The fact that the guinea-pig method has found widespread recognition in various parts of the world is no doubt due to the availability and low cost of this laboratory animal. Most laboratories utilizing the guinea-pig method have their own stock colonies. Under these conditions the cost of the assay is decreased considerably.

In our experiments an attempt has been made to simplify the technique of this method by using the brachial vein rather than resorting to the recognized practice of the Knaffl-Lenz method of exposing the jugular vein. We have not used artificial respiration and have not opened the chest cavity of the guinea pig in the last stages of the assay. Where

large numbers of preparations are assayed annually such practices would be too time consuming.

Table VI reveals that the standard error in our series of assays lies between 6–8% with a mean standard error of 7.1%. Ten guinea pigs were used in each assay. In fifteen consecutive U. S. P. XII cat assays, the standard error of the assay ranged from 4–7.5% with a mean standard error of 5.5% when six to eight cats were used for each assay.

In our opinion, an extensive comparative study of a large number of digitalis preparations of various types should be conducted by both the guinea-pig method and the cat method. Only after such an investigation will one be able to determine how closely the potency estimates obtained by the two methods check.

CONCLUSIONS

1. Some of the factors causing variation in the guinea-pig method for the standardization of digitalis have been studied.

2. The lethal dose determined in the conventional manner was high for light guinea pigs and low for heavy animals. A correction was made for such sensitivity. This adjustment is called the size factor and for the guinea pig equals 0.64 ± 0.039.

3. Fasting, sex, and source of supply are without influence on the results of the assay by the guinea-pig method.

4. The adjusted lethal dose remained constant when 1:15 and 1:30 dilutions of digitalis were infused into the animals at a constant rate.

5. Ether and sodium phenobarbital yield approximately the same lethal doses for the samples of digitalis tested.

6. The coefficient of variation of this method in our hands ranged from 13.8% to 17.4%.

7. Five preparations of digitalis have been assayed by the guinea-pig method and have been compared with the U. S. P. XI one-hour frog method and the U. S. P. XIII intravenous cat method. Four of the five preparations show good agreement in the potency estimates between the cat and guinea-pig methods. The potency of the fifth assayed by the guinea-pig method yielded a result differing by more than 20 per cent from that obtained by the intravenous cat method.

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Book Review

Volumetric Analysis. Vol. II. Titration Methods. By I. M. KOLTHOFF and V. A. STENGER. Interscience Publishers, New York, 1947. xiii + 374 pp. 15 x 23 cm. Price $6.00.

The authors, in bringing this volume up to date, have given particular attention not only to the fundamental principles of volumetric analysis, but to the practicability and reliability of the methods included therein.

A particularly good discussion of indicators is included in the chapter on Acidimetry and Alkalimetry. This is followed by a terse presentation of the principles of Acid-Base displacement titrations, titrations involving hydrolytic precipitations or complex formation, special methods of Acidimetry and Alkalimetry, argentometric titrations, other precipitation methods, and the formation of slightly dissociated or complex compounds.—NEULON DEAHL.